

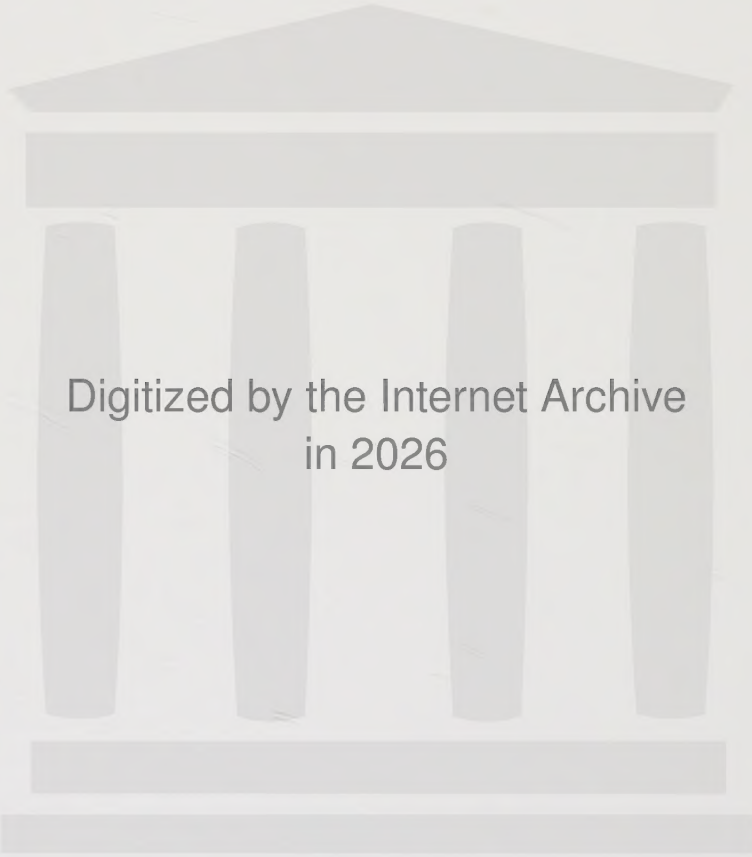
GUINEA PIG β_2 -MICROGLOBULIN

Studies on the isolation, structure and properties of
its two forms

Ragnhild Cigén



Lund 1984



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Organization LUND UNIVERSITY Department of Physiological Chemistry University of Lund P.O. Box 94, S-220 00 Lund, Sweden		Document name DOCTORAL DISSERTATION	
		Date of issue 1984-10-06	
		CODEN: LUMEDW/(MEMK-1019)/1-46/(1984)	
Author(s) Ragnhild Cig��n		Sponsoring organization	
Title and subtitle Guinea pig β_2 -microglobulin. Studies on the isolation, structure and properties of its two form.			
Abstract Guinea pig β_2 -microglobulin (β_{2m}) was isolated from urine of animals with sodium chromate induced proteinuria. The isolation scheme included gel filtrations, ion exchange chromatography and zone electrophoresis. Two forms of β_{2m} were detected differing in electrophoretic mobility and amino acid composition. Guinea pig β_{2m} was very similar to β_{2m} homologues from other species, as judged from physico-chemical and sequence studies. The structural difference between the two forms was demonstrated to be situated in the C-terminal end of the protein. The "acidic" form has asparagine as C-terminal amino acid and is one residue (lysine) shorter than the "basic" form. Both forms were detected in urine from inbred animals and in serum from outbred animals. Only the "basic" form was detected on membranes from liver tissue. The results were interpreted as that one of the forms is modified after translation and thus, does not depend on genetic polymorphism. An association between β_{2m} and the guinea pig Class I molecules (GPLA-B) was demonstrated by serological techniques. The ability of guinea pig β_{2m} , or its homologues, to interact with sera from other species was investigated. Similar results were obtained for all homologues. They bind to high molecular weight fractions in serum through exchange of preexisting β_{2m} in these complexes. This indicates that the binding structures on β_{2m} and the β_{2m} -binding serum molecules must be evolutionary well conserved and therefore, most probably consist of structures of critical functional importance. When the two guinea pig β_{2m} forms were allowed to bind to heterologous sera a difference in binding capacity was noted. Almost twice as much "basic" as "acidic" β_{2m} was bound. This points to that the C-terminal end of guinea pig β_{2m} is of importance in the interaction with other molecules.			
Key words β_2 -microglobulin, guinea pig, purification, properties, structure, C-terminal amino acids, two forms, distribution, serum, urine, liver membranes, GPLA antigens, heterologous interactions			
Classification system and/or index terms (if any)			
Supplementary bibliographical information			Language English
ISSN and key title			ISBN 91-7222-775-3
Recipient's notes		Number of pages 46	Price
		Security classification	

Distribution by (name and address)

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Date August 13, 1984

GUINEA PIG β_2 -MICROGLOBULIN
STUDIES ON THE ISOLATION, STRUCTURE AND PROPERTIES
OF ITS TWO FORMS

AKADEMISK AVHANDLING

som för vinnande av medicine doktorsexamen kommer att
försvaras offentligen i Hörsal B, Institutionen för
Medicinsk Kemi, Kemicentrum, Sölvegatan 39, Lund,
lördagen den 6 oktober 1984, 10.00

Av

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From the Department of Physiological Chemistry
University of Lund, Sweden

GUINEA PIG β_2 -MICROGLOBULIN

Studies on the isolation, structure and properties of
its two forms

Ragnhild Cigén

Lund 1984

To All My Little Friends

*Klokt kan människan handla
om hon beaktar tre ting:
Själlobesinning - det är det ädlaste
Följa mästare - det är det lättaste.
Söka sig fram på nya vägar -
det är det svåraste*

(Kung Fu-tse)

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LIST OF PAPERS

This thesis is based on the following papers referred to by Roman numerals in the text.

- I. Cigén R., Ziffer J.A., Berggård B., Cunningham B.A. & Berggård I. (1978) Guinea pig β_2 -Microglobulin. Purification, properties, and partial structure. *Biochemistry*, 17, 947-955.
- II. Cigén R. (1984) Structural difference between the two forms of guinea pig β_2 -microglobulin and their occurrence in inbred guinea pig strains. Submitted for publication.
- III. Björck L., Cigén R., Berggård B., Löw B. Berggård I. (1977) Relationships between β_2 -microglobulin and alloantigens coded for by the major histocompatibility complexes of the rabbit and the guinea pig. *Scand. J. Immunol.* 6, 1063-1069.
- IV. Cigén R. (1983) Distribution of the two forms of guinea pig β_2 -microglobulin originally detected in urine. *Molec. Immunol.* 20, 1385-1391.
- V. Lögdberg L., Cigén R. & Björck L. (1981) Binding of β_2 -microglobulin by heterologous sera. *Scand. J. Immunol.* 13, 237-243.
- VI. Cigén R. (1984) Difference in binding capacity between structurally different guinea pig β_2 -microglobulin forms and heterologous sera. Submitted for publication.

ABBREVIATION LIST

β_2m	- β_2 -microglobulin
CPA	- Carboxypeptidase A
CPB	- Carboxypeptidase B
CPA/B	- CPA contaminated with CPB
CPY	- Carboxypeptidase Y
DEAE	- Diethylaminoethyl
DFP	- Diisopropylphosphofluoridate
DNS-Cl	- 5-Dimethylamino-1-naphtalenesulfonyl-chloride
Fab'	- Monovalent antigen binding fragment of an immunoglobulin molecule
F(ab') ₂	- Bivalent antigen binding fragment of an immunoglobulin molecule
MHC	- Major Histocompatibility Complex
GPLA	- MHC of guinea pig
HLA	- MHC of man
H-2	- MHC of mouse
PMSF	- Phenylmethylsulfonylfluoride
SDS	- Sodium dodecyl sulfate
SDS-PAGE	- SDS-polyacrylamide gel electrophoresis

INTRODUCTION

In 1964 Ingemar Berggård published the first note on a small, globular, previously unknown plasma-protein, later named β_2 -microglobulin (β_2m) (Berggård, 1964). A few years later he described the purification procedure and a thorough characterization of β_2m , which was shown to consist of a single polypeptide chain of 11,800 mol. wt. with a characteristic intramolecular disulfide bond (Berggård and Bearn, 1968).

To isolate β_2m , Ingemar Berggård used urine from patients with tubular dysfunctions. Small plasma proteins (Mol. wt. < 50 000) are filtered through the glomerular membrane and then reabsorbed and catabolized in the cells of the proximal tubulus (cf. Strober and Waldman, 1974). When the reabsorption mechanism is damaged as in Wilson's disease, chronic cadmium poisoning or acute renal allograft rejection, large quantities of low molecular weight plasma proteins are found in the urine. Ingemar Berggård, who had analysed polysaccharides and proteins in normal urine (Berggård, 1961), early realized the benefit of "tubular urine" for the isolation of low molecular weight plasma proteins. Using such urine he was able to isolate, apart from β_2m , the previously unknown retinol-binding protein (RBP) (Peterson *et. al.*, 1971) and α_1 -microglobulin (Ekström *et. al.*, 1975). These proteins are nowadays subject for intensive research in various areas.

In the beginning, β_2m was mainly regarded as a model protein for studying kidney physiology. When its structural homology to immunoglobulin light chains was shown in 1972 (Smithies and Poulik, 1972b) β_2m gained interest from immunologists all over the world. In order to get a better understanding of β_2m , it became obvious to isolate β_2m -homologues from animals. In this thesis, the interest is focused mainly on β_2m in the guinea pig.

Purification and structure of β_2 m and its animal homologues are discussed in more detail in "GUINEA PIG β_2 -MICROGLOBULIN - PRESENT INVESTIGATION" and later in this chapter. In " β_2 -MICROGLOBULIN - A PRESENTATION" β_2 m refers to human β_2 m unless otherwise stated.

Distribution, synthesis and metabolism

Distribution

β_2 M has been detected in various biological fluids. Thus, apart from serum and urine, the presence of β_2 m has been demonstrated in colostrum, saliva, cerebrospinal, seminal and amniotic fluid and in ascites and pleural effusions (cf. Berggård *et. al.*, 1980 and Karlsson *et. al.*, 1980b). The normal serum concentration is about 1.5 mg/l (Evrin *et. al.*, 1971). There is no sex difference but the serum level increases slightly with age (Evrin and Wibell, 1972). The structural relationship of β_2 m to immunoglobulin domains (see below) initiated the search for β_2 m on lymphocyte surfaces. Peterson *et. al.* (1972) demonstrated that leukocytes bind 125 I-labeled antibodies against β_2 m. The presence of β_2 m on lymphocyte surface was clearly shown by lymphocytotoxicity and immunofluorescence studies (Poulik, 1973). The number of β_2 m-molecules on human lymphocytes has been estimated to be in the order of 10^5 (e.g. Evrin and Pertoft, 1973). In the guinea pig, rat and rabbit the number is somewhat lower (Björck *et. al.*, 1979).

It is believed that most somatic cells express β_2 m on the cell surface. Apart from studies with lymphocytes, information concerning cell surface expression and tissue distribution of β_2 m is rather limited and sometimes contradictory (Bhan *et. al.*, 1980; Baekkeskov *et. al.*, 1981; Chung-Park *et. al.*, 1980; Forsum and Malmnäs Tjernlund, 1977; Governa and Biguzzi, 1976; Lautenschlager *et. al.*, 1984; Ooi *et. al.*, 1977).

Mature erythrocytes and certain tumour cells lack β_2 m on the cell surface (Evrin and Pertoft, 1973; Nilsson *et. al.*, 1974; Pious *et. al.*, 1976; Weiss *et. al.*, 1981). In the Daudi cell-line an inactive messenger RNA explains the absence of β_2 m (de Préval and Mach, 1983; Rosa *et. al.*, 1983). Mouse

embryonic carcinoma cells (used as a model for normal early embryonic cells) are also reported not to express mouse β_2m (Dubois *et. al.*, 1976) although they do, if they are allowed to differentiate *in vitro* (Morello *et. al.*, 1982). In contrast, β_2m is detected on two-cell mouse embryos as shown by Sawicki *et. al.* (1981).

The occurrence of β_2m and transplantation antigens on spermatozoa is a matter of conflicting opinions (Andersson *et. al.*, 1982; Fellous *et. al.*, 1976; Law and Bodmer, 1978). It seems as if the presence of β_2m is dependent on the stage of development of the spermatozoa, as judged from studies in the rat (Söderström *et. al.*, 1982).

The presence of β_2m on trophoblasts in the placenta has been much discussed. It would be easy to explain the immunological acceptance of the fetus by the uterus by the absence of the transplantation antigens and thereby also β_2m (see below) on the trophoblasts. Initial reports also supported this hypothesis. In more recent studies, in mouse, β_2m and transplantation antigens have been found on the trophoblast subpopulation closest to the maternal circulation (cf. Searle, 1982). Small amounts of β_2m -messenger RNA have recently been detected in certain murine trophoblast cell clones (Tanaka *et. al.*, 1983).

Synthesis

Bernier and Fanger (1972) were the first to demonstrate the synthesis of β_2m by lymphocytes. This finding was soon extended to include cells and cell lines of other histogenic origin (cf. Nilsson *et. al.*, 1974). It is nowadays believed that most cells produce β_2m . Interferon increases the synthesis of β_2m in lymphoid cells (e.g. Fellous *et. al.*, 1981; Heron *et. al.*, 1978).

β_2m is encoded by a single gene per haploid genome. The mouse β_2m -gene consists of four coding blocks with non-coding regions in between (Parnes and Seidman, 1982). β_2m is initially synthesized with an N-terminal signal-peptide of 19 amino acids that is cleaved off before β_2m leaves the cell (Lingappa *et. al.*, 1979; Ploegh *et. al.*, 1979). In man, the β_2m gene lies on the long arm of chromosome 15 (Goodfellow *et. al.*, 1975; Faber *et. al.*, 1976). The discovery of allelic variants of mouse β_2m has facilitated the genetic mapping of β_2m . Thus, mouse β_2m is coded from chromosome 2 and the gene is closely linked to the locus of the minor histocompatibility complex,

H-3. Intriguing recent results suggest the identity of β_2m and its gene product(s) (cf. Michaelson, 1983; Margulies *et. al.*, 1983).

Metabolism

β_2m is synthesized at a rate of 0.13 mg/hour and kg bodyweight (Karlsson *et. al.*, 1979a). The half-life in the intravascular compartment is reported to be a little less than 2 h (Vincent *et. al.*, 1980). It is eliminated by the kidneys where β_2m is filtered through the glomerular membrane and then taken up by the tubulus cells by pinocytosis and degraded as judged from studies in rat, in the lysosomes (Maunsbach and Christiansen, 1974). Less than 0.1% (1.12 mg/24 h) of the β_2m escapes in the urine, but large amounts of β_2m are found in the urine of patients with tubular dysfunctions (Peterson *et. al.*, 1969). In patients with impaired glomerular filtration rate the serum concentration of β_2m rapidly increases. It has also been claimed that the serum β_2m level is more sensitive to reductions in the glomerular filtration rate than is serum-Creatinin (Wibell *et. al.*, 1973).

β_2 -Microglobulin and antigens of the Major Histocompatibility Complex

In 1973-74 the non-covalent association of β_2m to cell surface HLA-A antigens was discovered. This was shown biochemically (Grey *et. al.*, 1973; Nakamuro *et. al.*, 1974; Peterson *et. al.*, 1974) and by cocapping and cytotoxicity experiments (Neauport-Sautes *et. al.*, 1974; Østberg *et. al.*, 1974; Poulik *et. al.*, 1973; Solheim and Thorsby, 1974).

HLA-A antigens are human antigens encoded from the genetic region called the major histocompatibility complex. This complex is found on chromosome 6 in man and chromosome 17 in mouse (Fig. 1), and the MHC appears to be present in all vertebrates. The complex encodes for three classes of molecules that are involved in the recognition of and immune response to foreign molecules. Class I molecules, a family of extremely polymorphic cell surface molecules, serve as target molecules for cytotoxic T lymphocytes and participate in reactions like acute allograft rejection. A cell with a foreign molecule, for instance a cell transformed by virus, will be destroyed by a cytotoxic T lymphocyte, but only if both cell types involved carry the same set of Class I molecules. This is called MHC restriction. Class II molecules, also polymorphic, are involved in the regulation of the immune response, for instance to help it, and do also show MHC restriction in their actions. Class I molecules are found on most nucleated cells while Class II molecules

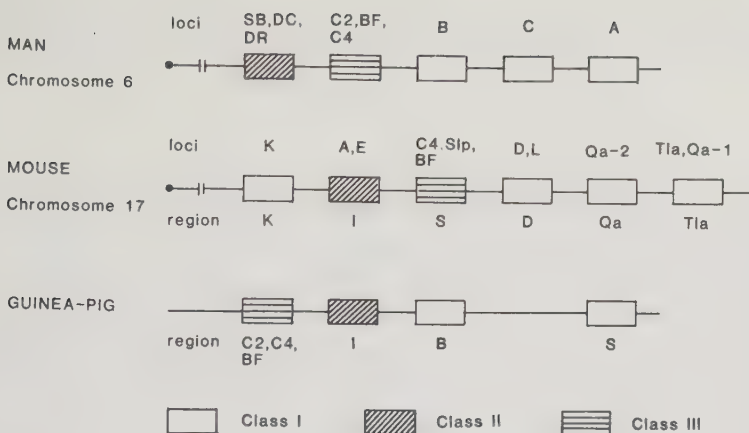


Fig. 1. Genetic organization of the major histocompatibility complexes of man, mouse and guinea pig (Shevach, 1980; Steinmetz and Hood, 1983). (● centromere).

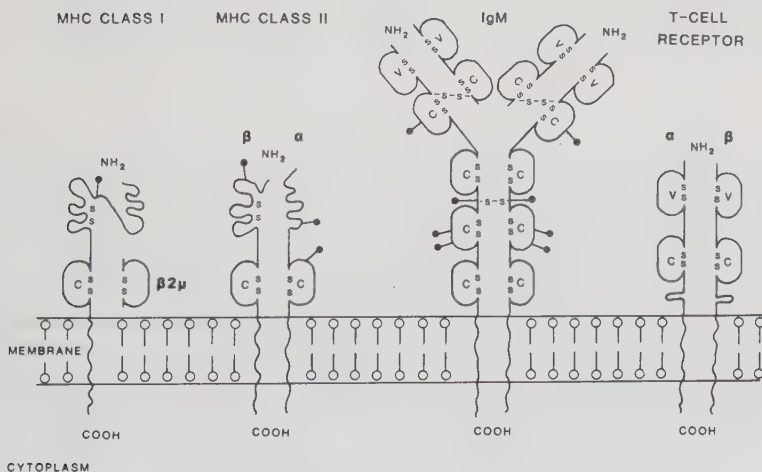


Fig. 2. Membrane proteins belonging to the "superfamily" of immunoglobulin-like molecules. The localization of β_2 -microglobulin in Class I molecules is demonstrated. Note the recurring domain structure in all molecules (modified from Kaufman *et. al.*, 1984 and Saito *et. al.*, 1984).

$\beta 2\mu$ = β_2 -microglobulin.

have a more restricted tissue distribution and are found predominantly on B cells and macrophages. Class III molecules belong to the complement system and are not further considered (cf. Kaufman *et. al.*, 1984; Steinmetz and Hood, 1983).

Class I molecules in man and mouse

Structural information concerning the Class I molecules are obtained mainly from studies in man and mouse. In man the classical Class I molecules are called HLA-A, B and C antigens and in the mouse H-2K, D and L antigens. They are transmembrane glycoproteins consisting of two chains, of which the light chain, as stated above, is identical to β_2m . The heavy chain has a molecular weight of approximately 45 K daltons. It carries the polymorphic characteristics and the carbohydrate. The polypeptide is folded into three extracellular and one intracellular domain with a stretch of hydrophobic amino acids in between that anchors the chain in the cell membrane (Fig. 2). The alloantigenic determinants are located on the two outmost domains. (Coligan *et. al.*, 1981; Lopez de Castro *et. al.*, 1979; Orr *et. al.*, 1979; Vega *et. al.*, 1984; Yokoyama and Nathenson, 1983). Mouse β_2m is non-covalently associated to the third extracellular domain (closest to the cell membrane) and β_2m has possibly also interaction sites to the second domain (Fig. 2) (Yokoyama *et. al.*, 1983). Human β_2m is most probably situated in a similar way in the HLA-A, B and C antigens (Vega *et. al.*, 1984).

Additional Class I molecules are the TL and Qa lymphocyte differentiation antigens best known from studies in the mouse. They are less polymorphic than the H-2K, D and L antigens and have a more limited tissue distribution. TL antigens are found on thymocytes in certain mouse strains and on some leukemic cells while Qa antigens are absent from thymocytes but are detected on T and B cells (cf. Steinmetz and Hood, 1983). These antigens are also membrane proteins with a heavy chain of 40-45 K daltons and a light chain that is β_2m (Michaelson *et. al.*, 1977; Østberg *et. al.*, 1975; Rothenberg and Triglia, 1981; Stanton and Hood, 1980; Vitetta *et. al.*, 1975).

Homologues to TL and Qa antigens have, by tissue distribution and biochemical analysis, also been suggested in man (Gazit *et. al.*, 1980, 1984; Knowles and Bodmer, 1982), guinea pig (Schwartz *et. al.*, 1978) and rat (Stock and Günther, 1982).

Class I molecules in the guinea pig

In the guinea pig, due to the genetic characteristics of the inbred animals available, interest has focused mainly on the Class II molecules. The information concerning Class I molecules is thus sparse.

In an original work by Sato and de Weck (1972), antigens, later designated GPLA-B.1, B.2, B.3, B.4 and S antigens, were identified. These were shown serologically by antisera obtained from cross-immunizations with lymphoid cells of randomly bred guinea pigs kept in a closed colony. Biochemical and tissue distribution studies indicated that these antigens were homologues to the mouse H-2K and D antigens. They are glycoproteins consisting of two polypeptide chains of 40 and 12 K daltons (cf. Shevach, 1980). The smaller chain was identified as β_2m serologically (Björck *et al.*, 1977a) and biochemically (Schwartz *et al.*, 1978). Tryptic peptide mapping and partial N-terminal sequence analysis demonstrate homologies between these antigens and to Class I antigens of other species (Rose *et al.*, 1979; Schwartz *et al.*, 1980).

The putative guinea pig TL antigens show marked homology to GPLA-B.1 and S antigens and have a tissue distribution of that of mouse TL antigens. However, no polymorphism has yet been detected in the guinea pig (Surrat *et al.*, 1983).

Additional molecules associated with β_2 -microglobulin

β_2m has been reported to associate with a mouse plasma-substance which resembles the H-2 antigen although it lacks alloantigenic reactivity (Kvist and Peterson, 1978; Natori *et al.*, 1976; Ramanathan *et al.*, 1982). This substance appears to be genetically controlled by the MHC (Kvist *et al.*, 1980b). Once it was believed that this molecule represented the gene product of an unusual H-2 related gene, lacking the coding sequence for the membrane associated amino acids and found only in liver tissue (Cosman *et al.*, 1982). Recently this gene product was identified and recognized in mouse serum. It associates with β_2m but is apparently not identical to the mouse plasma substance described above (Maloy *et al.*, 1984).

In a few studies the association of β_2m and tumour-specific antigens have been demonstrated (e.g. Malley *et al.*, 1979). Such findings support the "altered self" hypothesis that explains how a cell mediated immune response

is elicited by the recognition of the foreign antigen together with Class I molecules. It is noteworthy that β_2m is also associated with the serologically defined male-specific H-Y antigen (Fellous *et. al.*, 1978).

Structural homologies between β_2 -microglobulin and other members of the immunoglobulin-like "superfamily"

β_2M was recognized as an immunologically interesting protein when Smithies and Poulik in 1972 demonstrated that the N-terminal sequence of β_2m showed marked homologies to the constant region of immunoglobulin heavy chains.

The immunoglobulins consist of several domains (Edelman *et. al.*, 1969) (Fig. 2) and it is believed that the immunoglobulin genes have evolved by gene duplications and recombinations of an ancestral gene, large enough to code for one domain (Hill *et. al.*, 1966; Singer and Doolittle, 1966). The size of β_2m , about 100 amino acids, and its intra-chain disulfide loop of 56 amino acids together with the amino acid sequence and the later shown immunological cross-reactions to immunoglobulins strongly demonstrate the close relationship between β_2m and immunoglobulins (Berggård and Bearn, 1968; Cunningham *et. al.*, 1973; Gottlieb *et. al.*, 1977; Vincent and Revillard 1980). It has been suggested that β_2m may represent a free immunoglobulin domain (Peterson *et. al.*, 1972) and the question whether the β_2m -gene represented the ancestral immunoglobulin gene was raised (Smithies and Poulik, 1972b).

The domain structure is not unique to immunoglobulins and β_2m as structural studies of Class I (HLA) and Class II MHC antigens demonstrated a few years ago (Fig. 2). Sequence homologies to β_2m are most prominent in the β_2m -binding domain in Class I antigens (Larhammar *et. al.*, 1981; Orr *et. al.*, 1979; Trägårdh *et. al.*, 1979). Also the TL- and Qa Class I antigens are homologous to the classical Class I antigens (Soloski *et. al.*, 1982; Yokoyama *et. al.*, 1981). Very recently the amino acid sequence of the two chains of a mouse T-cell receptor was deduced from cDNA sequences and indeed these chains also contain the characteristic domain structure (Saito *et. al.*, 1984) (Fig. 2). The cell surface receptor for polymeric immunoglobulin A and immunoglobulin M (an Fc receptor), as well as differentiation antigen Thy-1, a glycoprotein found on some rodent cells, also belong to this "immunoglobulin superfamily" with sequence homologies and the polypeptide arranged in domains (Campbell *et. al.*, 1979; Mostov *et. al.*, 1984).

The homologies between β_2m and the above mentioned proteins are not limited to the final gene-product. Studies of the genes, the organization of exons and introns, demonstrate such homologies between immunoglobulins and MHC-antigens that the theory of a common evolutionary origin, and with the β_2m -gene as the candidate for the ancestral gene, is strongly supported (cf. Cushly and Owen, 1983 and Kindt, 1981).

These theories have prompted the search for β_2m and " β_2m -like" molecules in lower vertebrates and invertebrates. Preliminary candidates for such molecules have been detected (e.g. Shalev *et. al.*, 1981).

Functional aspects

The exact physiological role of β_2m is yet far from clear. Some clues to an eventual function have nevertheless been obtained.

It seems that β_2m is necessary for the intracellular transport and the cell surface expression of newly synthesized heavy chains of HLA-A,B and C antigens. This was initially indicated by Arce-Gomez *et. al.* (1978) and later confirmed by several authors (Owen *et. al.*, 1980; Ploegh *et. al.*, 1979; Sege *et. al.*, 1981) in that the β_2m -deficient human Daudi cell line is not able to express the heavy chain on the cell surface although it is synthesized and present intracellularly.

The importance of β_2m in keeping the heavy chain in an alloantigenic active conformation has also been discussed. The heavy chain of HLA-A,B and C antigens quickly loses its alloantigenic characteristics due to changed conformation when the two chains are separated (Krangel *et. al.*, 1979; Lancet *et. al.*, 1979; Owen *et. al.*, 1980). In the mouse, however, the H-2 heavy chain is reported to have alloantigenic reactivity when it is not associated with β_2m (Dobberstein *et. al.*, 1979; Kvist *et. al.*, 1977; Rogers *et. al.*, 1979).

β_2m has cytophilic properties and binds to cell surfaces (Andersson *et. al.*, 1975; Hester *et. al.*, 1979; Lögdberg *et. al.*, 1981; Sege *et. al.*, 1979) possibly by exchange of preexisting β_2m associated with Class I antigens. The functional significance of this is still unclear. The binding of β_2m to cells, serum-substances and purified Class I antigens will be discussed in more detail in "PRESENT INVESTIGATIONS".

The finding that β_2m in aggregated form binds to the M protein in certain streptococcal strains (Björck *et. al.*, 1981) raises intriguing questions for the understanding of the host - parasite relationship.

Many investigators have tried to elucidate the functional role of β_2m from the effects of β_2m or its antibody, in various biological assay systems. Many of these studies have been reviewed recently (Berggård *et. al.*, 1980) and only a few points will be summarized here.

The homology between β_2m and the Fc portion of immunoglobulin G (IgG) have suggested them to share some properties. Like the C_H2 domain of IgG β_2m is able to fix complement (Björck *et. al.*, 1984; Painter *et. al.*, 1974). β_2M promotes macrophage-rosetting of sheep red blood cells - a property also shared by the IgG C_H3 domain (Painter *et. al.*, 1974). β_2M also induces enhanced expression of Fc receptors for IgG on B lymphocytes (Birch *et. al.*, 1979). Antibodies against β_2m inhibit lymphocyte proliferation induced by cells, mitogens or soluble antigens as well as cell-mediated cytotoxicity (Bach *et. al.*, 1973, 1976; Lindblom *et. al.*, 1974; McMichael *et. al.*, 1980; Saxena *et. al.*, 1982; Solheim, 1974, Tomanari *et. al.*, 1982; Vincent *et. al.*, 1975). The antibodies themselves are found to be mitogenic to lymphocytes (Möller and Persson, 1974; Solheim, 1974). In addition, antibodies against β_2m have been shown to prolong heart allograft survival in the rat (Björck *et. al.*, 1977b). Thus, β_2m seems to be involved both in the inducing and effector phases of the immune system, although its precise role is far from clear.

Only a few studies have been performed with antibodies against guinea pig β_2m , but also here mitogenic properties of the antibodies on T cells have been recorded as also their inhibitory effects on mitogen or antigen induced lymphocyte activation (Lögdberg *et. al.*, 1979a; Yamachita *et. al.*, 1979).

Clinical aspects

The literature about β_2m in medicine is growing very fast, and a thorough survey of this subject is not the aim of this thesis. Therefore, only a brief summary will be made. Review articles and symposia are found in Karlsson *et. al.*, 1980b; *Pathologie Biologie*, 1978; Poulik, 1979.

A wealth of studies have dealt with the urinary β_2m excretion and serum- β_2m levels in renal disorders. The major findings have already been related. Because of an increased turnover rate, increased levels of β_2m in various biological fluids are found in inflammatory and autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE), Sjögren's syndrome etc. In some cases, especially in SLE, autoantibodies against β_2m have been detected (e.g. Revillard *et. al.*, 1979). Many, more or less, fruitless attempts have been made to correlate the serum- β_2m level to stages and prognosis of various neoplastic conditions. Maybe more interesting are the findings of a heterogenous cell surface expression of β_2m and associated Class I heavy chain antigen on cells in breast carcinoma and cutaneous malignancies (Mauduit *et. al.*, 1983; Sidky and Walker, 1984; Weiss *et. al.*, 1981). In multiple myeloma, however, the serum- β_2m level seems to correlate well to tumour mass and prognosis (e.g. Bataille *et. al.*, 1984). If the serum- β_2 level also will be useful, as has been indicated, in monitoring suspect cases of the much discussed acquired immune deficiency syndrome (AIDS) awaits further studies (e.g. Zolla-Pazner *et. al.*, 1984).

GUINEA PIG β_2 -MICROGLOBULIN - PRESENT INVESTIGATION

An ultimate aim in the research of β_2m is to determine and understand the physiological role of the protein. In order to do so, working on animal models with animal homologues to β_2m is a prerequisite. This thesis describes the purification and properties of the guinea pig β_2m . As two forms of guinea pig β_2m were detected, the distribution and the structural differences of these forms were studied as also their ability to interact with other molecules.

Purification (I)

Human β_2m was originally isolated from urine from patients with renal tubular dysfunctions that are found in disorders like chronic cadmium-poisoning and Wilson's disease (Berggård and Bearn, 1968). A similar approach was used in my isolation of the guinea pig β_2m homologue. Proteinuria in guinea pigs was induced by subcutaneous injections of sodium chromate. This agent is known to cause tubular malfunctions in rat (Balazs and Roepke, 1966) and has been successfully used in the purification of rat (Lögdberg *et. al.*, 1979b) and rabbit β_2m (Björck and Berggård, 1981).

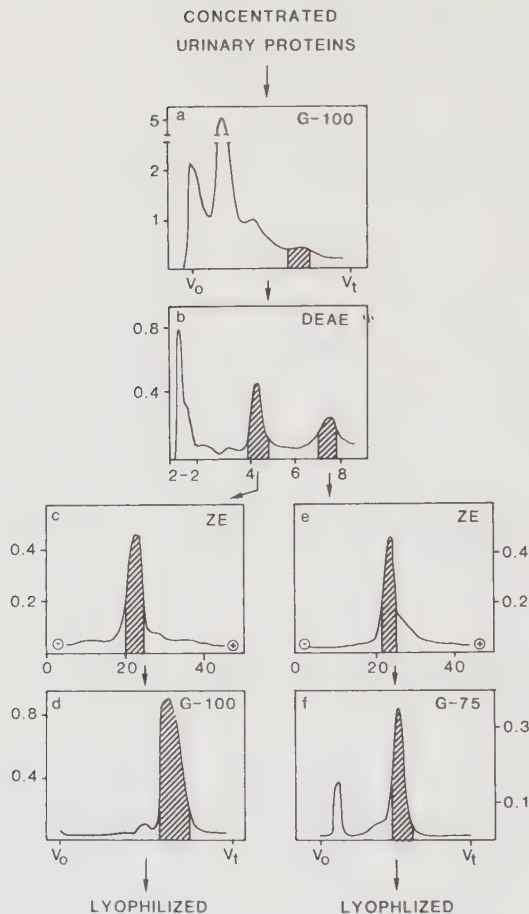


Fig. 3. Purification of the two forms of guinea pig β_2m . Representative graphs from different preparations have been selected and show the absorbance (280 nm) plotted against elution volume (a, d, f), conductivity ($m\Omega^{-1}\cdot cm^{-1}$) (b) or fraction number (c, e). Urine from animals with sodium chromate induced proteinuria was used as starting material and concentrated by ultrafiltration. The β_2m -containing fractions (hatched areas) were pooled and used for further purification. Gel filtrations were performed on Sephadex G-100 (a, d) or G-75 (f) in 0.02 M Tris-HCl, pH 8.0, with 0.15 M NaCl and 0.02% NaN₃. In the DEAE-ion exchange chromatography (b) 0.04 M Tris-HCl, pH 8.0, was used as starting buffer. Proteins were eluted with a linear NaCl gradient, 0-0.1 M, in the same buffer. Zone electrophoresis were run in 0.01 M borate buffer, pH 9.0 (c, e).

The β_{2m} content in normal guinea pig urine is 0.05-0.08 g/l (R. Cig n, unpublished), slightly less than reported in man (0.10 g/l) (Peterson *et. al.*, 1969). The daily excretion is about 1 mg. About a four-fold increase of the β_{2m} excretion is induced by the sodium-chromate treatment (R. Cig n, unpublished). This is far less than is the case in the rabbit or the rat where a 100- and 400-fold increase, respectively, have been reported (Bj r ck and Bergg rd, 1981; L gdberg, 1982). The reason for these interspecies' differences in tubular susceptibility to sodium chromate is unknown.

The purification of guinea pig β_{2m} included ultrafiltration of urine, gel filtration on Sephadex G-100, DEAE-cellulose ion exchange chromatography, zone electrophoresis on Pevicon blocks and finally, a second gel filtration on Sephadex G-100 (Fig. 3). The various buffers used are indicated in the legend to Fig. 3. When the purified β_{2m} appeared homogenous on polyacrylamide gel electrophoresis, the protein was used to prepare an antiserum for tracing β_{2m} in later preparations. The recovery range was 9-16% of the major or "basic" form, see below).

Guinea pig β_{2m} has also been isolated by two other groups. Stevenson *et. al.* (1978) also used urine from sodium chromate treated guinea pigs. They followed essentially the above described isolation scheme, although the electrophoretic step was left out. Wolfe and Cebra (1980) used potassium chromate as the nephrotoxic agent and ammonium sulfate precipitation as an initial purification step followed by various gel filtrations.

Apart from man, guinea pig, rat and rabbit β_{2m} also the dog homologue, the first animal β_{2m} to be detected, was isolated from urine (Smithies and Poulik, 1972a). Urine is, however, not the unique source for β_{2m} purification. Thus, human β_{2m} has also been isolated from lymphocyte culture media (Nakamuro *et. al.*, 1973) and colostrum (Cejka *et. al.*, 1976).

The most prominent finding during the β_{2m} isolation was the discovery of two β_{2m} forms, major and minor, in the ion exchange chromatography experiment. The minor form, later designated "acidic", constituted about 25% of total β_{2m} . This form was further purified as the major or "basic" form (Fig. 3), but in some cases the final gel filtration was performed on Sephadex G-75 rather than G-100 due to difficulties in separating the acidic β_{2m} from a contaminant. Variants of urinary β_{2m} were at that time also known from man (Hall *et. al.*, 1977) and have later also been encountered in rat (L gdberg

et. al., 1979b) and rabbit (Björck and Berggård, 1981). Allelic variants of β_2m have also been isolated from mouse urine (Graf *et. al.*, 1982). Bovine β_2m has been purified from milk (Groves and Greenberg, 1977) and mouse β_2m from liver and spleen membrane extracts (Natori *et. al.*, 1975). Serum has been used to isolate chicken (Winkler and Sanders, 1977) and turkey β_2m (Lillehoj *et. al.*, 1982).

Composition and properties (I, VI)

In Table I some of the physico-chemical properties of guinea pig β_2m (basic form) and its human, rabbit and rat homologues are presented. The combined data of the sedimentation coefficient, Stoke's radius, molecular weight and frictional ratio suggest that guinea pig β_2m is a compact, globular protein very much alike the other homologues. The homology also includes an intra-molecular disulfide loop of similar size in human β_2m and immunological cross reactions with the human, rabbit and rat counterparts (Björck and Berggård, 1981; Lögdberg *et al.*, 1979; R. Cigén, unpublished). Guinea pig β_2m has a concentration dependent tendency to self associate. This was noted as an asymmetry in the β_2m -peak in the final gel filtrations of the purification (Fig. 3d, f), and also in the results from the ultracentrifugation experiments. Self association has also been reported for bovine β_2m that initially was isolated as a tetramer, lactollin, from milk (Groves and Greenberg, 1977).

The mobility on agarose electrophoresis (pH 8.6) and the amino acid composition of the guinea pig β_2m forms were consistent with their elution characteristics in the ion exchange chromatography. On electrophoresis, the basic form migrated slightly slower and the acidic form slightly faster than human β_2m . The amino acid composition (Table II) revealed that the basic form contained one lysyl-residue more than the acidic form. In table II the composition was based on the assumption that guinea pig β_2m contained 100 amino acids, like, at that time believed, human β_2m (Cunningham *et. al.*, 1973). A recalculation on 99 or 98 amino acids (see below) does not alter the number of any amino acid residue. The number of glycine residues in the acidic form has varied somewhat (3-4) between preparations and it is not believed that this form contains one glycyl-residue more than the basic β_2m . Despite the differences in amino acid composition, the two forms showed immunological identity in double immunodiffusion tests. A close immunological relationship was also ascertained by radioimmunoassay, where

Table I. Physico-chemical properties of β_2 -microglobulin in guinea pig, rabbit, rat and man

	Guinea pig β_2 -microglobulin ^a	Rabbit β_2 -microglobulin ^b	Rat β_2 -microglobulin ^c	Human β_2 -microglobulin ^d
Sedimentation coefficient, S_{20}^0	1.6 S	1.65 S	1.6 S	1.6 S
Stoke's Radius, r_s	1.6 nm	1.6 nm	1.6 nm	1.6 nm
Apparent diffusion coefficient, D_{20}	133 $\mu\text{m}^2\text{s}^{-1}$	137 $\mu\text{m}^2\text{s}^{-1}$	130 $\mu\text{m}^2\text{s}^{-1}$	133 $\mu\text{m}^2\text{s}^{-1}$
Partial specific volume, $\bar{v}$	0.733 ml/g	0.732 ml/g	0.734 ml/g	0.727 ml/g
Molecular weight				
By sedimentation equilibrium ultracentrifugation		11,000	11,700	11,600
From sedimentation and diffusion constants	11,000	10,900		
By gel chromatography in 6 M Gdn-HCl ^e	11,700		12,300	
By SDS-PAGE	11,500	11,800	11,800	
From amino acid composition	11,415 ^f	11,655	11,750	11,728
Frictional ratio, f/f_0	1.12	1.11		1.16
Absorption coefficient at 280 nm (pH 7.0), ($\text{M}^{-1}\text{cm}^{-1}$)	1.52×10^4	1.61×10^4	1.42×10^4	1.98×10^4
Isoelectric point at 5°C	6.7	6.0	7.4	5.8

a Data from paper I unless otherwise indicated and refers to the basic guinea pig $\beta_2\text{m}$ form.

b Data from Björck and Berggård (1981).

c Data from Kvist *et al.*, (1980 a) and Lögdberg *et al.*, (1979 b).

d Data from Berggård and Bearn (1968), Berggård *et al.*, (1980), Karlsson *et al.*, (1974). Molecular weight from amino acid composition is a recalculation based on the corrected sequence (Parker and Strominger, 1982).

e Human β_2 -microglobulin was used as a reference protein. Gdn-HCl = Guanidine-HCl.

f Based on 99 amino acids found in the sequence (Wolfe and Cebra, 1980).

plots of standard dilutions of either form of purified β_2m were parallel (VI).

Table II

Amino acid composition of both forms of guinea pig β_2 -microglobulin

	Basic guinea pig β_2 -microglobulin	Acidic guinea pig β_2 -microglobulin
Asp/Asn	13.0 (13)	13.1 (13)
Thr	3.1 (3)	3.2 (3)
Ser	8.8 (9)	9.1 (9)
Glu/Gln	9.8 (10)	10.2 (10)
Pro	7.1 (7)	7.0 (7)
Gly	3.4 (3)	3.7 (4)
Ala	4.2 (4)	4.4 (4)
$\frac{1}{2}$ Cystine	1.9 (2)	2.0 (2)
Val	8.7 (9)	8.7 (9)
Met	0.8 (1)	0.9 (1)
Ile	4.9 (5)	4.9 (5)
Leu	7.1 (7)	7.1 (7)
Tyr	4.0 (4)	3.6 (4)
Phe	5.0 (5)	5.0 (5)
His	4.9 (5)	5.0 (5)
Lys	8.1 (8)	7.0 (7)
Arg	3.1 (3)	3.2 (3)
Trp	1.9 (2)	(2) (2)
Total	99.8 (100)	100.1 (100)

Data taken from paper I.

Sequence analysis of guinea pig β_2 -microglobulin (I, II)

In paper I, 17 of the 18 N-terminal amino acids of the basic guinea pig β_2m were determined. This partial sequence was confirmed when the complete amino acid sequence was published a few years later (Wolfe and Cebra, 1980). Apart from an extra glutamate/glutamine residue, the amino acid composition of the basic form (Table II) is in good agreement with the complete sequence (Fig. 4).

The guinea pig β_2m sequence shows extensive homologies to other sequenced β_2ms (Fig. 4). The homology is most prominent to rabbit β_2m , where 74 of the 99 amino acid residues are identical (Gates *et. al.*, 1979). The number of identical residues to other β_2ms varies between 60 and 68 (Cunningham *et. al.*, 1973; Gates *et. al.*, 1979, 1981; Groves and Greenberg, 1982; Lögdberg, 1982; Parker and Strominger, 1982).

β_2m	10	20	30	40	50
GUINEA-PIG	V L H A P P V G V Y S P H P A E N G K N F I N C Y V S G F H P P Q I E V E L L Y N G K K I D N V E				
HUMAN	I Q R T - K I - - - - - S - - L - - - - - S D - - D - - D - E R - E K - -				
COW	I Q R P - K I - - - - - P - - - P - Y L - - - Y - - - - - I D - - - E - - K S - -				
RABBIT	- O R - - N - - - - - P - - L - - - - - D I - - - - V - - E - -				
RAT	I O K T - Q I - - - - - P - - P - L - - - Q - - - - - I - - - - - P - I -				
MOUSE	I O K T - Q I - - - - - P - - P - I L - - - T Q - - - H - - I Q M - - - - P K - -				
DOG	- Q - P - K I - - - - - P - - L - - - - - ? Z - - I D - - - D				
PIG	- A R P - K - - - - - P - Y L - ? - - - - - I D - - - E				
TURKEY	K I E - - I K				
	60	70	80	90	99
GUINEA-PIG	M S D L S F S K D W T F / L L V H A A F T P N D S D E Y S C R V S H I T L S E P K I V K W D P N K *				
HUMAN	H - - - - - S - - - Y Y T E - - T E K - - - A - - N - V - - Q - - - - R D M				
COW	Q - - - - - S - - - S - E - - D S K - - - - K - V - - E Q - R - - - R D L				
RABBIT	Q - - - - N - - S - - - T E - - - N K N - - - - K - V - - K - - M T - - - R D Y				
RAT	- - - - - S - - I - A - T E - - T E T - V - A - - K - V - - K - - T - T - - R D M				
MOUSE	- - - M - - - S - - I - A - T E - - T E T - T - A - - K - ^A _D S M A - - - T - Y - - R D M				

Fig. 4. Complete amino acid sequences of the β_2 -microglobulin homologues of guinea pig (Wolfe and Cebra, 1980), man (Cunningham *et al.*, 1973; Parry and Strominger, 1982) cow (Groves and Greenberg, 1982), rabbit (Gates *et al.*, 1979), rat (Lögberg, 1982) mouse (Gates *et al.*, 1981) and partial amino acid sequences of dog (Smithies and Poulik, 1972a), pig (Metzger *et al.*, 1982) and turkey β_2 -microglobulin (Lillehoj *et al.*, 1982).

The combined findings of the differences in electrophoretic mobility and amino acid composition between the two guinea pig β_2m forms prompted a structural study to localize the difference. One of our suggestions about the two forms in paper I was that the difference was situated in the C-terminal end of the protein. The sequence studies (Wolfe and Cebra, 1980) also supported this idea. Thus, the β_2m forms, after reduction and alkylation, were digested by carboxypeptidases Y and B. The released amino acids were dansylated and identified by thin layer chromatography on polyamide sheets. Fig. 5 summarizes the digestion results. The results were interpreted to mean that the basic form has lysine and the acidic form has asparagine as their respective C-terminal amino acids. As no lysine was found in the acidic digest but asparagine was detected, after the appearance of lysine in the basic digest, it was concluded that the acidic form is one amino acid residue shorter than the basic β_2m . Electrophoretic studies on SDS-PAGE

also supported this concept. The acidic β_2m had, reproducibly, a slightly higher mobility than the basic form,

DIGESTION- TIME (min)	GUINEA-PIG β_2 -MICROGLOBULIN		
	BASIC		ACIDIC
	CPA/B	CPY	CPY
0	—	—	—
15	—	—	(□)
30	■	—	□
60	■	N.D.	N.D.
120	■	■ □ ● ○	□ ● ○

Fig. 5. Digestion of basic and acidic guinea pig β_2m with carboxipeptidase A contaminated with carboxypeptidase B (CPA/B) and with carboxypeptidase Y. After dansylation, the digests were separated in two directions by solvents 1, 2 and 3 on polyamide sheets. (■) lysine; (□) asparagine; (●, ○) unidentified amino acids; N.D. not determined; Solvent 1 = 1.5 % formic acid; Solvent 2 = benzene-acetic acid, 9:1 v/v; Solvent 3 = ethylacetate-acetic acid-methanol, 20:1:1, v/v.

The orientation of the C-terminal amino acids in β_2m is unknown. Limited crystallographic and spectrometric studies together with comparisons with immunoglobulin domains have given a model for the secondary and tertiary structure of bovine and human β_2m . About half of the amino acid residues are apparently organized in seven strands of β -pleated sheets and a few residues in an α -helix structure. The β -strands and α -helix are believed to be folded as immunoglobulin domains (Becker *et. al.*, 1977; Cohen *et. al.*, 1980; Karlsson, 1974; Lancet *et. al.*, 1979; Poljak *et. al.*, 1974). The C-terminal residues, however, are apparently not part of any organized sub-structure and nothing is known about their eventual interactions with other residues. As the peptide-bonds of these residues were unavailable to

digestion unless reduced and alkylated it seems reasonable to believe that they are hidden from the surface of the protein.

The structural background to the variants of β_2m in man, rat and rabbit are still unknown (Björck and Berggård, 1981; Hall *et. al.*, 1977; Lögdberg *et. al.* 1979 b). In mouse the allelic variants depend on an amino acid substitution in position 85 (Gates *et. al.*, 1981).

Distribution (III, IV, V)

Incomplete studies suggest the presence of guinea pig β_2m in most tissues as judged by radioimmunoassay of homogenates made serum-free by perfusion (Cigén and Akerström, unpublished).

Cells

In paper III the association of β_2m to GPLA-B antigens (= MHC Class I molecules) on lymphocyte surfaces was established. Similar results had, at that time, been obtained in man (Grey *et. al.*, 1973; Nakamuro *et. al.*, 1973; Peterson *et. al.*, 1974) and mouse (Natori *et. al.*, 1974; Rask *et. al.*, 1974; Silver and Hood, 1974). It is nowadays believed that β_2m is part of MHC Class I molecules in all mammals. In paper III Fab' fragments of a goat antiserum against guinea pig β_2m were preincubated with lymphocytes and the cytotoxicity of an antiserum against alloanti-GPLA-B antigen was tested. No cytotoxic effect was recorded, indicating that the Fab' fragments sterically blocked the GPLA-B antigens - i.e. guinea pig β_2m is physically associated with the GPLA antigens on the cell surface. This conclusion was further supported in that the cytotoxic effect of alloanti-GPLA-B was abolished also when β_2m had been "removed" from the cell surface by "lyso-strip" experiments. In such experiments β_2m was first aggregated on the cell surface by successive incubations of the cells with F(ab')₂ fragments of goat-anti-guinea pig β_2m and rabbit-anti-goat-IgG. The aggregates were then taken up by the cells. β_2m and associated structures are "removed" from the surface. The subsequent lack of cytotoxic action of alloanti-GPLA indicate that the GPLA antigens are aggregated together with the β_2m molecule. Similar experiments failed to demonstrate any association between β_2m and Ia-antigens (Class II molecules) as once was suggested (Callahan *et. al.*, 1976). As previously related β_2m is, on the cell surface, associated also to the guinea pig homologues of the Class I differentiation antigens TL and Qa-2 (Schwartz *et. al.*, 1978).

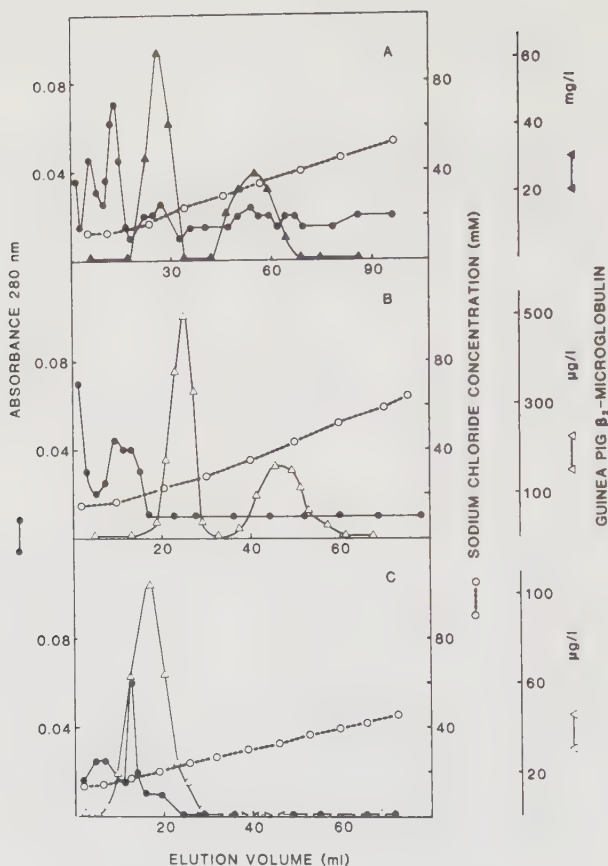


Fig. 6. Ion exchange chromatography of the combined and concentrated fractions of gel filtration of normal guinea pig urine (A), guinea pig serum (B), and solubilized guinea pig liver membranes (C). The samples were dialyzed against 0.02 M Tris-HCl, pH 8.0, and applied to columns (0.6 x 6.5 cm) of Sephadex-DEAE A-50, equilibrated with the same buffer. Total proteins, determined by the absorbance at 280 nm, (●—●) were eluted with a linear gradient of sodium chloride, 0-0.1 M (O—O). Guinea pig β_2 m was determined by single radial immunodiffusion (Δ — Δ) or a radioimmunoassay ($\blacktriangle$ — $\blacktriangle$).

Serum

The serum concentration of guinea pig β_2m is in the range of 3-7 mg/l (IV), somewhat higher than in man (Evrin *et. al.*, 1971). In serum guinea pig β_2m is found both in monomeric, "free", form and in high molecular weight complexes (IV, V). On Sephadex G-200 gel filtration the β_2m complexes elute at an approximate K_{av} of 0.33. This complex was not characterized in rat and mouse, where apparently similar complexes are found. The complexes, apart from β_2m , consist of glycoproteins antigenically or structurally related to the heavy chain of MHC Class I molecules (Kvist and Peterson, 1978; Maloy *et.al.*, 1984; Natori *et. al.*, 1976; Ramanathan *et. al.*, 1982; Vincent *et. al.*, 1981).

Distribution of the two β_2 -microglobulin forms (IV)

When guinea pig β_2m was purified, urine from outbred animals was used. It was impossible to say anything about the origin of the two forms. It could either depend on genetic polymorphism or on some kind of postsynthetic modification of one of the forms. Allelic variants of β_2m have long been looked for and are to date only found in the mouse (Goding and Walker, 1980; Michaelson *et. al.*, 1980).

To determine whether the two guinea pig forms of β_2m represent allelic variants or not, urine from inbred strains 2 and 13 was collected and analysed after separation on gel filtration and ion exchange chromatography. The results showed two distinct β_2m peaks. They were very similar to those presented in Fig. 6 a that shows the results from a corresponding ion exchange chromatography of urinary β_2m from an outbred animal and to results obtained from pooled urine from several outbred guinea pigs. Thus, the guinea pig β_2m forms apparently are not results of the expression of different β_2m genes but rather of posttranslational modification of a single gene product.

One possible origin of the two forms is that one of them may be a urinary degradation product. The easiest way to rule this out is to investigate their presence in serum. Pooled serum from several animals was ultrafiltered through a membrane that filters monomeric β_2m but retains most of the plasma proteins. In this way monomeric β_2m was separated from β_2m as associated with higher molecular weight complexes that could interfere with the interpretation of the results. After further purification on gel

filtration the serum- β_2m was analysed on ion exchange chromatography where two β_2m -peaks were detected in agreement with what earlier has been found in urine (Fig. 6a, b).

As much of the functional role of β_2m apparently involves its association to Class I molecules on cell surfaces, a detergent solubilized membrane fraction of perfused and serum-free liver tissue was analysed for the presence of the two guinea pig β_2m forms. The ion exchange chromatography results are presented in Fig. 6 c, and here only the basic form is detected.

To summarize, both β_2m forms are found in serum and urine, but only the basic β_2m on membranes from liver tissue. A convenient explanation of the origin of the forms is that an exopeptidase in serum releases the C-terminal lysine of the basic β_2m . It is, however, difficult to see how this could be accomplished. Unless reduced and alkylated, carboxypeptidases were unable to release any amino acid residue (II; Wolfe and Cebra, 1980). If this hypothesis holds true β_2m must, after digestion *in vivo*, regain most of its tertiary structure as the acidic form obviously reacts well with antibodies made against the native basic β_2m (I, II, IV, VI). Another possibility would be that the modification occurs intracellularly before the folding of the protein is complete.

Interactions between β_2 -microglobulin and other molecules (V, VI)

Evolutionary aspects on the interaction between β_2 -microglobulin and heterologous sera (V)

In 1976 Jones *et.al.* reported that a heavy chain of human Class I molecules could, in cell hybrids, be expressed on the cell surface together with mouse β_2m . This indicates that the binding surfaces of the involved molecules are evolutionary well conserved. In order to get further support for this hypothesis, the binding of guinea pig β_2m (basic form) as well as rat and human β_2m , to components in sera from other species (heterologous sera) was investigated (V).

β_2M was incubated in various mammalian sera at 37 $^{\circ}$ C for 1-2 days. The incubation mixture was then separated by gel filtration on Sephadex G-200 and the β_2m -content in the fractions determined by radioimmunoassay. Table III shows the incubation results with guinea pig β_2m . The peaks and

Table III. Binding of guinea pig β_2 -microglobulin by heterologous sera.

Serum	Recovered β_2 m in		
	Peak A	Peak B	Peak C
Man	(+)	(+)	++
Rhesus Monkey	(+)	(+)	+
Rat	(+)	-	+
Mouse	-	++	++
Rabbit	+	(+)	++
Cow	-	-	(+)
Horse	-	++	++
Goat	+	+	++

Data are taken from paper V. Guinea pig β_2 m was added to various sera to a final concentration of 2 μ g/ml. The mixtures were incubated 1 day at 37 $^{\circ}$ C and then chromatographed on columns of Sephadex G-200. The β_2 m content in the fractions was determined by radioimmunoassay. The β_2 m-containing peaks (A, B, C) were integrated to measure their total β_2 m content. - = not detected; (+) = β_2 m detected but peak not distinct; + = distinct β_2 m-peak containing < 10% of total β_2 m; ++ = β_2 m-peak containing > 10% of total β_2 m. Peak A corresponds to the void peak (peak K_{av} =0); peak B elutes between the two first protein peaks (K_{av} =0.08-0.15); peak C is detected around the third protein peak (K_{av} =0.32-0.42) (See also Fig. 7 where region I roughly coincides with peak B and region II with peak C).

the score indices are defined in the legend to the table. The elution profiles of the exogenously added, heterologous β_2 m were very similar to those of the endogenous β_2 m. For instance, when small amounts of rat β_2 m were added to guinea pig serum, the rat β_2 m eluted at the same positions as guinea pig β_2 m. When a larger quantity, however, of guinea pig β_2 m concomitantly was added to the guinea pig serum-rat β_2 m incubation, the rat β_2 m was recovered uncomplexed in its monomeric form. There also seemed to be a relationship between the substances in peak B and peak C. When peak B material from a horse serum-rat β_2 m incubation was re-chromatographed the β_2 m reappeared in the C peak. Lability of a mouse β_2 m-containing peak in the B region of mouse serum has previously been reported (Kvist and Peterson, 1978). The ability of β_2 m to bind to heterologous sera has also been described by Ramanathan *et al.* (1982) and Vincent *et al.* (1981).

The proposed mechanism for binding of heterologous β_2m to serum molecules is that added β_2m exchanges for preexisting homologous β_2m . This was first suggested when it was found that ^{125}I -rat β_2m incubated with rat serum eluted at the same positions as the β_2m already present in the serum (Lögdberg *et. al.*, 1979 b). An equilibrium association-dissociation exchange mechanism has been shown to explain the binding of β_2m to purified homologous or heterologous Class I antigens (Church *et. al.*, 1982; Graf *et. al.*, 1982; Hyafil and Strominger, 1979; Lögdberg *et. al.*, 1980; Schmidt *et al.*, 1981).

An exchange reaction with the β_2m associated with Class I molecules is also believed to be responsible for at least part of the β_2m -binding to homologous or heterologous cells (Bernabeu *et. al.*, 1984; Hester *et. al.*, 1979; Hyafil and Strominger, 1979; Kefford *et. al.*, 1984; Lögdberg *et. al.*, 1981; Schmidt *et. al.*, 1981). However, other β_2m -binding molecules have also been suggested (Sege *et. al.*, 1979; Sege and Peterson, 1980).

As various β_2m s are able to interact with molecules over the species barriers in apparently similar ways, the structural homologies can not be confined to the β_2m molecule alone. Also, the β_2m binding structure on the interacting molecules, in many cases shown to be the heavy chain of Class I molecules, must be evolutionary well conserved, implying that these structures are of importance, maybe vital, for the survival of the individual. Binding studies of this kind are also a valuable complement in evolutionary studies of the molecules in the immune system (e.g. Lögdberg and Björck, 1983).

Influence of the guinea pig β_2 -microglobulin C-terminal amino acid on the interaction with heterologous sera

The structures on the β_2m molecule responsible for the interaction with other molecules are largely unknown. Parker and Strominger (1983) recently reported that two of the tyrosine residues in human β_2m (Tyr-10 and -26) may be involved in the binding to the heavy chain of a HLA-B molecule. β_2M , in which these residues were iodinated, did not exchange into the HLA-B complex.

In order to elucidate whether the C-terminal end of guinea pig β_2m is of any importance in the protein's interaction with other molecules, studies

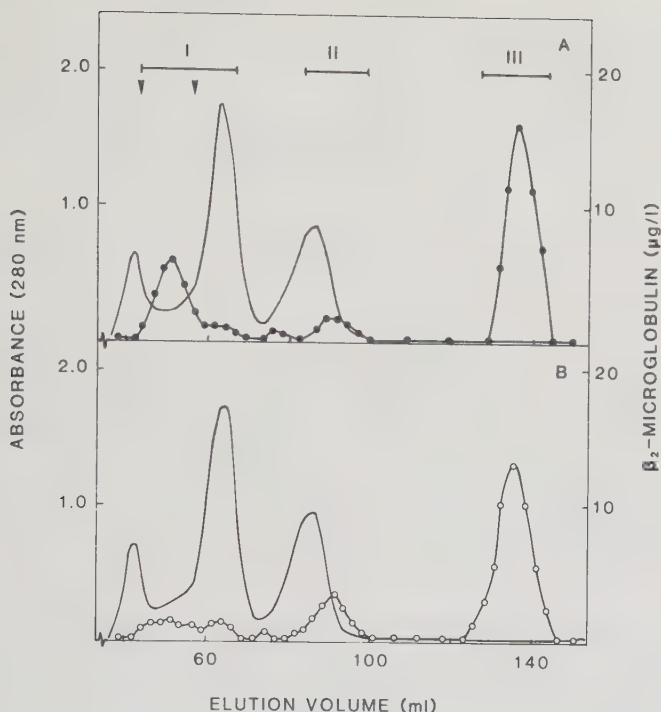


Fig. 7. Sephadex G-200 chromatography of 0.5 ml horse serum incubated for 12 h, 37⁰, with 1.0 μ g A) basic (●—●) or B) acidic (○—○) β_2 m. Total protein was determined by the absorbance at 280 nm (—) and β_2 m by radioimmunoassay.

were undertaken to quantitatively compare the ability of the two guinea pig β_2 m forms to bind to heterologous sera. Horse serum mixed with either form was chromatographed on Sephadex G-200 after 1-24 h of incubation. β_2 M, determined by radioimmunoassay, eluted in three regions defined in Fig. 7. The figure shows the β_2 m elution profiles of both forms after 12 h of incubation (region I coincides roughly with peak B in paper V; region II with peak C).

In region I more basic than acidic β_2 m was bound, regardless of incubation-time and in region II equal amounts or, after longer incubation periods, more acidic than basic β_2 m was detected. But, taken together, almost twice

as much basic as acidic β_2m was bound in all incubations. Also in mouse serum more basic than acidic β_2m was bound. It is known that β_2m -complexes in region I are labile and degrade to region II complexes (Kvist and Peterson, 1978; Ramanathan *et. al.*, 1982; V, VI). Thus it seems reasonable to believe, that region I, to which acidic β_2m is bound, decomposes more easily than those with basic β_2m , which would explain why more acidic than basic β_2m is detected in region II after longer incubation periods.

The difference in binding capacity between the two guinea pig β_2m forms most probably depends on the different C-terminal end. The C-terminal lysine residue, only found in the basic form, could be directly involved in a binding-site. Electrostatic forces have been reported to participate in the binding of human β_2m to cod serum glycoproteins (Lögberg and Björck, 1983). The lysine residue could also be of importance in retaining the tertiary conformation of β_2m necessary for its interactions with other molecules. More information concerning the tertiary structure of guinea pig β_2m and the binding molecules in horse serum is, however, needed to make more confirmative conclusions.

As related in a previous chapter, it is believed that the most conserved amino acids in β_2m are responsible for the binding to e.g. the heavy chain of Class I molecules. Therefore, it is somewhat puzzling that the C-terminal sequence of guinea pig β_2m is unique to this animal. This would argue in favour for the hypothesis that the lesser binding capacity of the acidic β_2m depends on an altered conformation rather than loss of a direct binding-site. The number of C-terminal sequences in various β_2m s is still limited (Fig. 4) (Cunningham *et. al.*, 1973; Gates *et. al.*, 1979; 1981; Groves and Greenberg, 1982; Lögberg, 1982).

The nature of the β_2m -binding molecules in horse serum are unknown. In analogy with what has been shown in rat and mouse, one could speculate in that these molecules contain subunits antigenically or structurally related to the horse's Class I molecules (Kvist and Peterson, 1978; Maloy *et. al.*, 1984; Natori *et. al.*, 1976; Ramanathan *et. al.*, 1982; Vincent *et. al.*, 1981). If this holds true, the lesser binding of acidic β_2m to horse serum is in accordance with the finding that no acidic β_2m was detected on membranes from liver tissue (IV). Whether this lack of acidic β_2m on membranes is due to a low affinity of acidic β_2m to heavy chains of Class I molecules is not established and binding studies to purified Class I molecules would be of interest.

GENERAL SUMMARY

1. Guinea pig β_2 -microglobulin (β_{2m}) was purified from animals with sodium chromate induced proteinuria. The isolation procedure included gel filtrations, ion exchange chromatography and zone electrophoresis. It was concluded from physico-chemical properties, amino acid composition and presence of an intramolecular disulfide bond that guinea pig β_{2m} is a compact, globular protein with a molecular weight of 11,500 daltons. Guinea pig β_{2m} also showed extensive homologies to β_{2m} s from other species.
2. The most prominent finding during the isolation of guinea pig β_{2m} was the discovery of two forms, major (basic) and minor (acidic). Immunological identity was demonstrated between the forms but they differed in electrophoretic mobility and amino acid composition. The difference in the amino acid sequence was, by digestion with carboxypeptidases, localized to the C-terminal end of the protein. The acidic form has asparagine as the C-terminal amino acid and is one amino acid (lysine) shorter than the basic β_{2m} . Both forms were detected in urine and serum. Also urine from inbred guinea pigs contained both forms. Only the basic β_{2m} was detected on membranes from liver tissue. Thus, it is probable that one of the forms is a post-translational modification of the other form.
3. Guinea pig β_{2m} is associated, on the cell surface, with Class I molecules of the major histocompatibility complex. This was demonstrated by cytotoxicity experiments. The results were in accordance with what has been found in other species.
4. Guinea pig β_{2m} is able, as also human and rat β_{2m} , to bind to high molecular weight complexes in serum from various species. The binding characteristics, determined by the elution profile of β_{2m} on gel filtration, after incubation of β_{2m} with serum, were similar for all tested homologues of β_{2m} . Moreover, the exogenously added β_{2m} was eluted at the same positions as the endogenous β_{2m} . This indicates that the binding structures not only on β_{2m} but also on the binding serum-molecules are evolutionary well conserved.

5. The ability of the two guinea pig β_2m forms to bind to heterologous sera was compared. The results demonstrated that almost twice as much basic as acidic β_2m was bound. It is believed that the C-terminal lysine of the basic β_2m form is of importance for the binding, not directly, but by stabilizing the binding tertiary structure of the protein. Results from analysis of the primary C-terminal structure indicate that the C-terminal is situated in the interior of the protein.

ACKNOWLEDGEMENTS

I would like to thank and express my deep gratitude to

- the late professor Ingemar Berggård. Ingemar introduced me to β_2 -microglobulin and the scientific world. β_2 -Microglobulin is still my daily companion, sometimes loved, sometimes not. Ingemar's personal qualities of ambition, honesty and carefulness, also influencing his sincere interest in science, still inspire me.
- Lennart Lögdberg, Bo Åkerström, Barbro Berggård and Lars Björck for fruitful collaboration, friendship and never ceasing discussions.
- Mss Britt-Marie Cedergren, Inga-Maria Frick, Cecilia Frostgård, Britta Lindholm, Maj-Britt Nilsvik, Iris Olsson-Heenen, Elisabeth Persson, Cecilia Svensson and Boel Wieslander for expert technical and secretarial assistance during various phases of the work.
- Bo Thorén for helping me with everyday matters and Mss Britt Nilsson and Ingegerd Nilsson for cleaning all my tubes.
- Mss Gesa Johnson and Gunvor Lårusson for their efficient typing of the thesis and manuscripts and Ms Aniela Szulczynski for providing beautiful photographs.
- Professor Bengt Borgström and all other friends and colleagues at the Department of Physiological Chemistry for advice, chats and the lending of various items and chemicals whenever needed.
- Peter for eliminating many mis-spellings and grammatical blunders in this thesis - and for being my husband!

Financial support was recieved from the Medical Research Council, the Medical Faculty at the University of Lund, Konung Gustav V:s 80-års fond, the Swedish Society of Medical Research, and the Royal Physiographic Society of Lund.

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APPENDIX: PAPER I-IV

Guinea Pig β_2 -Microglobulin. Purification, Properties, and Partial Structure[†]

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ABSTRACT: β_2 -Microglobulin has been purified from urine of guinea pigs treated with sodium chromate by a combination of ultrafiltration, gel chromatography, ion-exchange chromatography, and zone electrophoresis. Two forms of the protein, differing in net charge, were isolated. On Ouchterlony immunodiffusion analyses, the two forms appeared immunologically identical and cross-reacted with rabbit β_2 -microglobulin. At pH 8 to 9, however, the net charge of the major type was more positive than that of the minor type, and the amino acid composition of the major form contained 8 lysyl residues whereas that of the minor form contained only 7. The two proteins therefore may represent genetically distinct forms of β_2 -microglobulin. Alternatively, the minor component may be derived from the major protein by proteolysis or deamidation. The results of amino acid analyses and of experiments

with starch gel electrophoresis in urea indicate that both guinea pig proteins have a disulfide loop analogous to those characteristic of human β_2 -microglobulin and the immunoglobulin domains. The major form of the protein constitutes about three-fourths of the total urinary β_2 -microglobulin, and the amino acid sequence of the first 18 residues of this form is closely homologous to corresponding sequences in β_2 -microglobulins from other species, particularly the rabbit. Physicochemical characterization of the protein revealed a molecular weight of about 11 500, a sedimentation coefficient of 1.6 S, a Stokes' radius of 1.6 nm, and an isoelectric point of 6.6. The molecule has a low frictional ratio (1.12) indicating a compact, globular shape. The protein has a tendency to self-associate at high concentrations.

Human β_2 -microglobulin is a small protein produced by various types of cells (Nilsson et al., 1973). It occurs both bound to cell surfaces (Peterson et al., 1972; Poulik, 1973) and in free form in various body fluids (Berggård & Bearn, 1968; Berggård, 1965). The protein is of special interest because it is structurally related to the immunoglobulins (Smithies & Poulik, 1972a; Peterson et al., 1972) and is homologous to the constant domains of these proteins (Peterson et al., 1972; Cunningham et al., 1973; Cunningham, 1976). Furthermore, recent work has shown that it occurs associated with human HLA histocompatibility antigens (Nakamuro et al., 1973; Grey et al., 1973; Peterson et al., 1974).

Studies of β_2 -microglobulin from other species have become important in order to gain more insight into the structural, evolutionary, and functional relations of this protein to histocompatibility antigens, to other cell surface structures, and to immunoglobulins. During recent years, β_2 -microglobulins have been isolated from dog (Smithies & Poulik, 1972b), rabbit (Berggård, 1974; Cunningham & Berggård, 1975), mouse

(Natori et al., 1975; Appella et al., 1976), rat (Poulik et al., 1975), chicken (Winkler & Sanders, 1977), and cow (Groves & Greenberg, 1977). The mouse homologue of β_2 -microglobulin is associated not only with the major H-2 histocompatibility antigens (Rask et al., 1974; Silver & Hood, 1974) but also with the closely related thymus leukemia (TL) antigens (Östberg et al., 1975; Vitetta et al., 1975) and Qa-2 antigens (Michaelson et al., 1977).

Structural and genetic variants of β_2 -microglobulin from any species could be particularly valuable in determining its biological activity, its interactions with specific cell surface molecules, and the organization of β_2 -microglobulin genes in mammalian chromosomes. No genetic variant of β_2 -microglobulin has been unambiguously identified. Variant forms of the protein, however, have been described. For example, lactolin, a crystalline protein of molecular weight 43 000, isolated from milk (Groves et al., 1963), is composed of subunits of molecular weight 12 000 that represent the bovine homologue of β_2 -microglobulin (Groves & Greenberg, 1977), and two forms of human β_2 -microglobulins, differing in isoelectric point, have been detected (Hall et al., 1977).

This report describes the preparation of guinea pig β_2 -microglobulin. The protein was obtained in two forms that differ in isoelectric point and amino acid composition. A partial physicochemical and structural characterization has been carried out, particularly of the major form which resembles the human protein in many properties, but differs from human β_2 -microglobulin in that it aggregates at high concentrations.

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Some preliminary results of this investigation have been reported in a previous communication (Berggård, 1976).

Materials and Methods

Urines and Sera. Concentrates of urinary proteins were prepared from five to seven 24-h urine specimens collected from each of 10 to 21 outbred guinea pigs treated with a single subcutaneous dose of sodium chromate (15 mg/kg of body weight of a solution containing 20 mg/mL). Only male guinea pigs were used and they were kept in metabolic cages with one animal per cage. In most experiments, some animals died before the end of a collection period. The surviving animals were given repeated injections after periods of rest. To each collecting vessel 0.5 mL of 5% sodium azide was added as a preservative. When a 24-h collection was terminated, the sodium azide concentration was adjusted to 0.1%. The urine specimens were pooled, adjusted to pH 6.5, centrifuged, tested for protein with Uristix strips (Ames Co., Slough, England), and then stored at -20°C until the end of the collection period. Tests for protein were usually negative in normal urine but positive after sodium chromate administration with maximum proteinuria (2+ to 3+) during the first 3 to 4 days after injection.

Sera were obtained from healthy guinea pigs which were bled from the carotid artery. After removal of the clot, the sera were stored at -20°C until used.

Antisera. Antisera were raised in one goat and two rabbits against the major form of guinea pig β_2 -microglobulin and in two rabbits against the minor form of this protein (see below). The immunization procedures have been described (Berggård & Bearn, 1968; Björck et al., 1977). The goat antiserum appeared specific, and one of the rabbit antisera practically specific for the isolated proteins when tested on immunoelectrophoresis against varying amounts of concentrated urinary proteins from guinea pigs treated with sodium chromate. Antisera against rabbit β_2 -microglobulin were prepared as described (Björck et al., 1977).

Other Materials. DEAE-cellulose 2S-3H was obtained from Serva Feinbiochemica, Heidelberg, Germany; Pevikon C-870 was from Kema Nord AB, Stockholm, Sweden; partially hydrolyzed starch was from Connaught Laboratories, Toronto, Canada; polyamide thin layers were from Schleicher and Schuell, Keene, N.H.; aminopeptidase M was from Rohm & Haas, Darmstadt, Germany; carrier ampholytes (Ampholine) were from LKB-Produkter AB, Bromma, Sweden; and $^{23}\text{S}_2$ -in. dialysis tubing was from Union Carbide Corp., Chicago, Ill. Human and rabbit β_2 -microglobulins were prepared as described (Berggård & Bearn, 1968; Berggård, 1974). Sephadex G-100 and Sepharose CL-6B were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and prepared according to the instructions supplied. All other chemicals were reagent grade or of the best quality available.

Protein Determinations. Protein concentrations were measured by the modified Folin method of Lowry et al. (1951) or by reading the absorbance at 280 nm. The major form of guinea pig β_2 -microglobulin was used as the standard in both procedures. In the Folin method, guinea pig β_2 -microglobulin gave about the same absorbancy as an equal amount of human IgG. The absorption coefficient of guinea pig β_2 -microglobulin at 280 nm and its light-absorption spectrum were determined in 0.1 M sodium phosphate buffer (pH 7.0) on a Gilford Model 222 spectrophotometer, after correction for ash and moisture content. Moisture was measured by drying to constant weight at 105°C and 0.05 mmHg.

Concentration of Proteins. Urine, sera, and protein solutions were concentrated by ultrafiltration with $^{23}\text{S}_2$ -in. dialysis

tubing from Union Carbide (Berggård, 1962). This tubing retains β_2 -microglobulin completely under the conditions used (Berggård & Bearn, 1968). Urine was concentrated 40- to 90-fold and centrifuged, before purification of β_2 -microglobulin.

Electrophoretic Methods. Preparative zone electrophoresis in blocks of Pevikon C-870, polyacrylamide gel electrophoresis in a discontinuous buffer system, and starch gel electrophoresis in 8 M urea and formate buffer (pH 3.0) were performed with previously reported techniques (Peterson & Berggård, 1971). A 0.1 M sodium borate buffer (pH 8.9) was used for preparative zone electrophoresis (Berggård & Bearn, 1968). Protein samples analyzed by starch gel electrophoresis were dissolved in 0.2 M Tris-HCl buffer (pH 8.0) or in the same buffer containing 8 M urea. Some of these samples were reduced with 0.1 M 2-mercaptoethanol for 60 min at room temperature and thereafter alkylated with 0.12 M iodoacetamide for 30 min.

Agarose gel electrophoresis was carried out in 75 mM sodium barbital buffer (pH 8.6) containing 2 mM calcium lactate, essentially as described by Johansson (1972).

Polyacrylamide gel electrophoresis in slabs with sodium dodecyl sulfate was performed according to Neville (1971) using the GE-4 apparatus from Pharmacia Fine Chemicals, Uppsala, Sweden. The running gel had a pH of 9.2 and an acrylamide concentration of 16% with an acrylamide/bisacrylamide ratio of 97:3. Human IgG heavy chain, chicken egg albumin, human IgG light chain, bovine ribonuclease, and human β_2 -microglobulin were used as molecular weight markers, assuming values of 50 000, 43 000, 23 500, 13 700, and 11 800, respectively.

Isoelectric Focusing. Analytical isoelectric focusing was conducted at 5°C in a 110-mL column (LKB-Produkter AB, Bromma, Sweden) essentially as described by Vesterberg (1971). Carrier ampholyte solutions of pH range 4 to 8 were used. The pH gradient was stabilized by a sucrose gradient from 0 to 50%. Focusing proceeded for 24 h at a constant power of 5.6 W and a current from 4 to 2 mA. The effluent from the column was analyzed for pH, for protein by reading the absorbance at 280 nm, and for β_2 -microglobulin by single radial immunodiffusion.

Immunodiffusion Techniques. Immunodiffusion in gel according to Ouchterlony, immunoelectrophoresis, and single radial immunodiffusion were performed as previously reported (Berggård & Bearn, 1968).

Ultracentrifugation. Sedimentation velocity and equilibrium experiments were conducted at 20°C in a Beckman Model E analytical ultracentrifuge, equipped with an RTIC temperature control unit and an electronic speed control device (Beckman Instruments, Palo Alto, Calif.). The protein samples were examined in 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. Standard 12-mm double sector cells with sapphire windows were used. The centrifuge was operated at 60 000 or 48 000 rpm for the sedimentation velocity determinations. The sedimenting boundary was recorded every 4 min with the phase plate schlieren optics. Calculations were done as described by Schachman (1957).

Sedimentation equilibrium experiments were carried out with the meniscus depletion technique of Yphantis (1964). Recordings were made with Rayleigh interference optics.

Determination of Molecular Weight by Gel Chromatography. Analyses were made on a Sepharose CL-6B column (120×1.5 cm), equilibrated with 0.05 M sodium acetate buffer (pH 4.8) containing 6 M guanidine hydrochloride, Gdn-HCl¹ (Mann and Fish, 1972). Heavy and light chains of

¹ Abbreviations used: Gdn-HCl, guanidine hydrochloride; Pth, phenylthiohydantoin.

human IgG, bovine albumin, chicken egg albumin, equine myoglobin, and human β_2 -microglobulin were used as reference proteins. Guinea pig β_2 -microglobulin and the reference proteins were reduced for 2 h in 0.2 M Tris-HCl buffer (pH 8.0) containing 6 M guanidine hydrochloride and 0.02 M dithioerythritol. Thereafter, iodoacetamide was added to a concentration of 0.05 M and the samples were left to alkylate for 30 min in the dark.

Estimation of Stokes' Radius. Stokes' radius (r_s) was measured at room temperature by chromatography on a Sephadex G-100 column (107 $\times$ 0.9 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl. The details of the experimental procedure have been described (Karlsson et al., 1972). Bovine albumin ($r_s = 3.6$ nm), bovine chymotrypsinogen ($r_s = 2.2$ nm) and human β_2 -microglobulin ($r_s = 1.6$ nm) were utilized to calibrate the column. The equation given by Laurent & Killander (1964) was used for calculation of Stokes' radius from the gel chromatography data.

Calculations of the Apparent Diffusion Coefficient, of the Molecular Weight by the Svedberg Equation, and of the Frictional Ratio. Stokes-Einstein's equation (Gosting, 1956) was used to calculate the apparent diffusion coefficient ($D_{20,w}$) from Stokes' radius. The molecular weight was determined from the diffusion coefficient and the sedimentation coefficient by Svedberg's equation. The frictional ratio (f/f_0) was computed from the sedimentation coefficient and the molecular weight derived from the amino acid composition of the protein. A partial specific volume for the calculations was estimated from the amino acid composition as described by Cohn & Edsall (1943). The formulas used have been reported by Svedberg & Pedersen (1940).

Amino Acid Analysis. Quantitative amino acid analyses were carried out on a Durrum Model D-500 amino acid analyzer (Durrum, Palo Alto, Calif.). Protein samples of about 0.5 mg were hydrolyzed in 6 M HCl at 110 $^{\circ}$ C for 24 and 72 h (von Hofsten et al., 1965). Half-cystine was measured as cysteic acid after performic acid oxidation (Hirs, 1967). Half-cystine was also determined as carboxymethylcysteine. For these analyses, samples of protein (5 mg/mL) were reduced for 60 min in 0.2 M Tris-HCl buffer (pH 8.0) containing 6 M Gdn-HCl and 0.01 M dithioerythritol. The samples were alkylated at room temperature for 15 min in the dark by addition of a 15% molar excess (over reagent - SH) of iodoacetamide. Amino acid analyses were performed after exhaustive dialysis against distilled water at 4 $^{\circ}$ C, followed by lyophilization and hydrolysis. Tryptophan was estimated spectrophotometrically as described by Edelhoch (1967).

Analysis of Free Sulfhydryl Groups. These were determined by carboxymethylation followed by analyses for carboxymethylcysteine on the amino acid analyzer. Protein samples (2.5 mg/mL) were treated for 15 min in the dark with 2 mM iodoacetamide in 0.2 M Tris-HCl buffer (pH 8.0) containing 6 M guanidine hydrochloride. Thereafter the samples were dialyzed exhaustively against distilled water at 4 $^{\circ}$ C, lyophilized, and submitted to amino acid analysis.

Sequence Analyses. Half-cystines were reduced with dithiothreitol in the presence of 6 M guanidine hydrochloride and alkylated with iodoacetamide (Waxdal et al., 1968). Excess reagents were removed by exhaustive dialysis against distilled water. Amino-terminal sequence analysis on 100 nmol of the reduced and alkylated protein was performed using the automated method of Edman & Begg (1967) in a Beckman Model 890 C sequencer with the Quadrol double cleavage program (Beckman Instruments, Palo Alto, Calif.). The thiazolanone amino acids were converted to their phenylthiohy-

TABLE I: Purification of the Major Form of Guinea Pig β_2 -Microglobulin.

Purification step	Total protein ^a (mg)	β_2 -Micro-globulin ^b (mg)	Yield (%)	Purification (fold)
Concentrated urinary proteins ^c	5900	73	100	1
First Sephadex G-100 chromatography	190	43	59	18
DEAE-cellulose chromatography	33	19	26	46
Zone electrophoresis	13	12	16	74
Second Sephadex G-100 chromatography	9	9	12	81

^a Determined by the Folin method in the first four steps and then spectrophotometrically from the optical density at 280 nm. ^b Measured by single radial immunodiffusion. ^c The urinary proteins were obtained from four to six 24-h specimens of urine from each of 21 guinea pigs treated with sodium chromate.

dantoin (Pth) derivatives using 1 M HCl and identified qualitatively using polyamide thin layers according to the method of Summers et al. (1973). Arginine (Yamada & Itano, 1966) and histidine were detected calorimetrically. Leucine was resolved from isoleucine by 18-h hydrolysis of the Pth-amino acid in 6 M HCl at 150 $^{\circ}$ C, followed by amino acid analysis using a Beckman Model 121 M amino acid analyzer (Beckman Instruments). Manual sequence analysis was carried out on 100 nmol of the reduced and alkylated protein using the dansyl-Edman technique of Gottlieb et al. (1970); identification was carried out using the thin-layer chromatography system of Weiner et al. (1972). Amino-peptidase M digestion was used to quantitate the results.

Results

Purification of Two Forms of Guinea Pig β_2 -Microglobulin. Two homologues of β_2 -microglobulin were obtained. One of the forms was present in the urine concentrates in about three times higher quantities than the other form (see below). Antisera were raised against the first preparations of β_2 -microglobulin and were used to trace and measure the protein during the following purifications.

The various isolation steps are described below and the protocol of a typical purification of the major form of the protein is summarized in Table I. All operations were carried out at 4 $^{\circ}$ C starting from concentrates of pooled urine. The yields of the major form varied between 9 and 16% of the total amount of β_2 -microglobulin found in concentrates of urine.

First Gel Chromatography on Sephadex G-100. Concentrated urinary proteins from guinea pigs treated with sodium chromate were chromatographed on Sephadex G-100 columns (113 $\times$ 8 or 104 $\times$ 5 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl and 0.02% Na₂S₂O₃. In early purifications, protein with the elution volume of human β_2 -microglobulin was taken to the next step (DEAE-cellulose chromatography). When antisera became available, the effluents from the columns were analyzed both for total protein by reading the absorbance at 280 nm and for guinea pig β_2 -microglobulin by single radial immunodiffusion. β_2 -Microglobulin was eluted from the Sephadex G-100 columns after most of the other urinary proteins (Figure 1) but did not appear as a peak in the absorbance at 280 nm.

Chromatography on DEAE-Cellulose. The pooled fractions from one or two Sephadex G-100 chromatographies were dialyzed against 0.04 M Tris-HCl buffer (pH 8.0) and applied

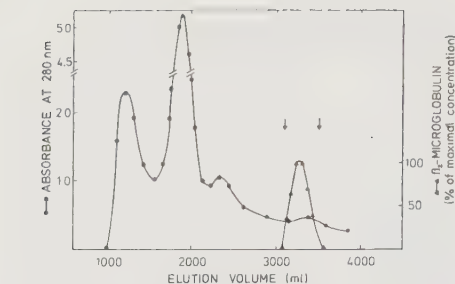


FIGURE 1: Chromatography on a Sephadex G-100 column (113 × 8 cm) of concentrated urinary proteins from 20 guinea pigs with renal damage induced by sodium chromate. The sample (2.6 g of protein in 18 mL) was eluted with a 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl and 0.02% NaN_3 . Fractions of 14.8 mL were collected at a flow rate of 44.4 mL per h. The distributions in the effluent of protein (●) and β_2 -microglobulin (▲) are shown. Fractions containing β_2 -microglobulin were combined as indicated by the arrows.

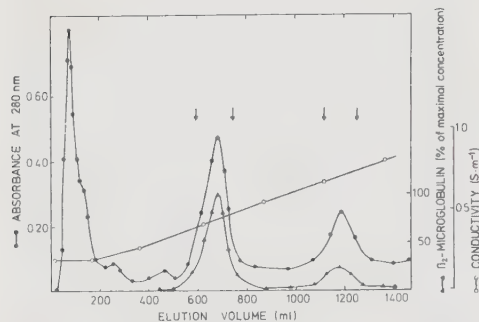


FIGURE 2: DEAE-cellulose chromatography of the β_2 -microglobulin-containing material from gel chromatography on Sephadex G-100. The sample (0.40 g of protein) was dialyzed against 0.04 M Tris-HCl buffer (pH 8.0) and applied to a column (13.0 × 3.5 cm) of DEAE-cellulose 23-SH equilibrated with the same buffer. The column was eluted in 0.04 M Tris-HCl buffer (pH 8.0) with a 2000-mL linear gradient of sodium chloride from 0 M to 0.1 M. Fractions of 6.2 mL were collected at a flow rate of 18.6 mL per h. The effluent was analyzed for protein (●), β_2 -microglobulin (▲), and conductivity (○). Eluates corresponding to a large and a small peak of β_2 -microglobulin were pooled as marked by the arrows.

to a DEAE-cellulose column that was equilibrated with the same buffer. Elution was performed with a linear gradient of sodium chloride (0 to 0.1 M). Figure 2 shows that three main protein fractions were obtained. The last material to be eluted emerged at about the same sodium chloride concentration as human β_2 -microglobulin. Therefore, only the protein present in this fraction was used for further purification in the initial work. The first antisera were prepared against a homologue of β_2 -microglobulin obtained from this material. With these antisera, we found that β_2 -microglobulin was present also in the intermediate fraction and in higher quantities. The ratio of the amounts of β_2 -microglobulin in the two fractions was about 1 to 3.

Zone Electrophoresis in Borate Buffer (pH 8.9). The two β_2 -microglobulin-containing fractions from DEAE-cellulose chromatography were concentrated separately and subjected to preparative zone electrophoresis in 0.1 M sodium borate buffer, pH 8.9. Material from one to four columns was used for each separation. Usually, only one distinct protein com-

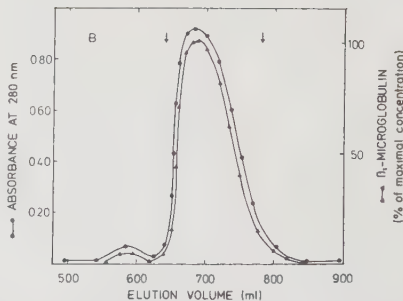
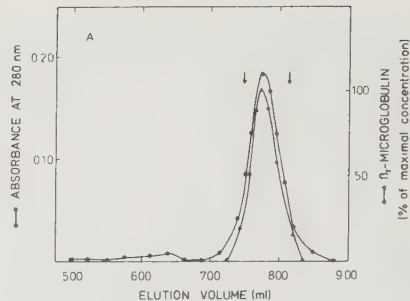


FIGURE 3: Gel chromatography on Sephadex G-100 of the major form of guinea pig β_2 -microglobulin previously purified by zone electrophoresis in borate buffer. The column (145 × 3 cm) was equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) containing 0.15 M NaCl and 0.02% NaN_3 . The distributions in the effluent of total protein (●) and β_2 -microglobulin (▲) are shown. The arrows indicate how the fractions were combined. (A) Thirteen milligrams of protein in 5.1 mL was applied. Fractions of 6.0 mL were collected at 20-min intervals. (B) Seventy milligrams of protein in 8.4 mL was applied. Fractions of 3.3 mL were collected at 20-min intervals.

ponent containing β_2 -microglobulin was observed after electrophoresis of the major or the minor fraction from DEAE cellulose. Some contaminants were removed, especially from the minor fraction. Most contaminants were found in the anodal direction from the main protein component. The minor fraction has a higher mobility than the major fraction; both fractions migrated toward the anode.

Second Gel Chromatography on Sephadex G-100. After concentration, the two β_2 -microglobulin fractions were separately chromatographed on a Sephadex G-100 column (145 × 3 cm). Moderate amounts of the major fraction (5–15 mg) gave symmetrical peaks with the elution volume of human β_2 -microglobulin (Figure 3A). In contrast, large amounts of this fraction (50–70 mg) gave asymmetrical peaks with significantly smaller elution volumes (Figure 3B). Only a slight purification was achieved by this final step (Figure 3A and Table I). A small amount of material containing β_2 -microglobulin appeared ahead of the main peak when relatively large amounts of the major fraction were chromatographed (Figure 3B).

On Sephadex G-100 chromatography, the minor β_2 -microglobulin fraction resolved into two protein components that were not completely separated from each other. The first component was a contaminant, whereas the second consisted of β_2 -microglobulin, as shown by single radial immunodiffusion analyses.

After each chromatography, the eluate corresponding to the

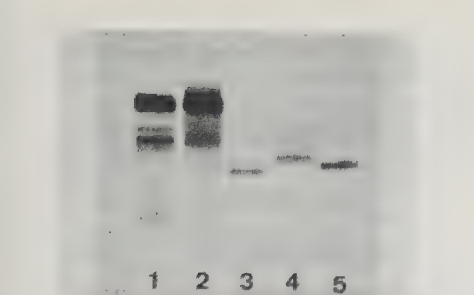


FIGURE 4: Agarose gel electrophoresis at pH 8.6 of serum from a normal guinea pig (1), concentrated urinary proteins from a guinea pig treated with sodium chromate (2), the major form of guinea pig β_2 -microglobulin (3), the minor form of guinea pig β_2 -microglobulin (4), and human β_2 -microglobulin (5). The anode is at the top.



FIGURE 5: Ouchterlony immunodiffusion analysis of the major form of guinea pig β_2 -microglobulin (1 and 4), the minor form of the protein (3 and 6), concentrated urine from a guinea pig treated with sodium chromate (2), and concentrated serum from an untreated guinea pig (5). The center well contained goat antiserum against the major form of guinea pig β_2 -microglobulin.

main part of the β_2 -microglobulin peak was dialyzed exhaustively against distilled, deionized water, and then lyophilized.

Purity and Charge Difference of the Two Forms of the Protein. The purity of the isolated protein was assessed by agarose gel electrophoresis and by polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate. Usually, only single protein bands were seen. The results of amino acid analyses and isoelectric focusing experiments also indicated purity (see below).

The charge difference between the two forms of guinea pig β_2 -microglobulin observed during the purification was confirmed by agarose gel electrophoresis and by polyacrylamide gel electrophoresis. The major form had somewhat lower mobility than human β_2 -microglobulin whereas the minor form migrated somewhat faster (Figure 4).

Immunodiffusion Analyses. On Ouchterlony immunodiffusion analyses using goat antiserum against rabbit β_2 -microglobulin, the two purified guinea pig proteins gave relatively weak precipitin lines that fused completely with each other and gave a reaction of partial identity with the strong precipitin line of rabbit β_2 -microglobulin. This observation indicates that both proteins are homologues of β_2 -microglobulin and rather closely related to the rabbit homologue of this protein.

Figure 5 shows that guinea pig serum and urine contain β_2 -microglobulin that gives reactions of identity with the purified major and minor forms of this protein when an antiserum

TABLE II: Amino Acid Composition^a of the Major and the Minor Form of Guinea Pig β_2 -Microglobulin.

Amino acid	The major form of the protein	The minor form of the protein
Asp	13.0 $\pm$ 0.4 (13)	13.1 $\pm$ 0.3 (13)
Thr ^b	3.1 $\pm$ 0.1 (3)	3.2 $\pm$ 0.1 (3)
Ser ^b	8.8 $\pm$ 0.3 (9)	9.1 $\pm$ 0.1 (9)
Glu	9.8 $\pm$ 0.2 (10)	10.2 $\pm$ 0.4 (10)
Pro	7.1 $\pm$ 0.2 (7)	7.0 $\pm$ 0.2 (7)
Gly	3.4 $\pm$ 0.2 (3)	3.7 $\pm$ 0.2 (4)
Ala	4.2 $\pm$ 0.1 (4)	4.4 $\pm$ 0.1 (4)
1/2-cystine ^c	1.9 $\pm$ 0.3 (2)	2.0 $\pm$ 0.2 (2)
Val ^d	8.7 $\pm$ 0.3 (9)	8.7 $\pm$ 0.3 (9)
Met	0.8 $\pm$ 0.1 (1)	0.9 $\pm$ 0.1 (1)
Ile ^d	4.9 $\pm$ 0.1 (5)	4.9 $\pm$ 0.2 (5)
Leu	7.1 $\pm$ 0.3 (7)	7.1 $\pm$ 0.2 (7)
Tyr ^b	4.0 $\pm$ 0.4 (4)	3.6 $\pm$ 0.5 (4)
Phe	5.0 $\pm$ 0.1 (5)	5.0 $\pm$ 0.2 (5)
His	4.9 $\pm$ 0.1 (5)	5.0 $\pm$ 0.3 (5)
Lys	8.1 $\pm$ 0.3 (8)	7.0 $\pm$ 0.3 (7)
Arg	3.1 $\pm$ 0.1 (3)	3.2 $\pm$ 0.1 (3)
Trp ^e	1.9 (2)	(2) ^f (2)
Total	99.8 (100)	100.1 (100)

^a Calculated on the basis of 100 amino acid residues per molecule. Except where noted, all figures are means $\pm$ SD obtained from analyses of four different preparations of each form of the protein, and each analysis is an average of one 24-h and one 72-h hydrolysis. The nearest integer values are given in parentheses. ^b Values obtained by extrapolation to zero-hour hydrolysis. ^c Measured as cysteic acid on three preparations of each form. ^d Seventy-two-hour hydrolysis value only. ^e Determined spectrophotometrically on two preparations of the major form. The average value is given. ^f Not determined.

against the major form is used. Reactions of identity between the two forms were found with all antisera directed against either form. One strong and one weak precipitin line were always seen (Figure 4). Two parallel arcs, one strong and one weak, were observed in immunoelectrophoretic experiments. We have observed this phenomenon with two precipitin lines or arcs also when other purified β_2 -microglobulins (human, rabbit, and rat) have reacted with their corresponding antisera. Therefore, and because both forms of guinea pig β_2 -microglobulin gave the same two lines, it is highly probable that both lines are given by β_2 -microglobulin.

Amino Acid Composition. The amino acid compositions of the two types of guinea pig β_2 -microglobulin are given in Table II. The recovery was about 95% (w/w). The calculations were based on the assumption that the two guinea pig proteins contain 100 residues per molecule, like human β_2 -microglobulin (Berggård & Bearn, 1968; Cunningham et al., 1973). The two forms seem to have the same composition except that the minor form contains one residue less of lysine. Close to integral numbers of residues were obtained for all amino acids with the exception of glycine, suggesting that the protein samples are homogeneous. The number of half-cystine residues in the major form was 1.9 per molecule when measured as cysteic acid after performic acid oxidation and 1.8 when measured as carboxymethylcysteine after reduction and alkylation in 6 M guanidine hydrochloride. No evidence for free sulfhydryl groups in this protein was obtained by analysis for carboxymethylcysteine after carboxymethylation in 6 M guanidine hydrochloride.

Evidence for Similar Disulfide-Linked Loops in Guinea Pig β_2 -Microglobulin and Its Human Counterpart. On starch gel electrophoresis in 8 M urea and formate buffer, the mobilities of both guinea pig proteins and human β_2 -microglobulin de-

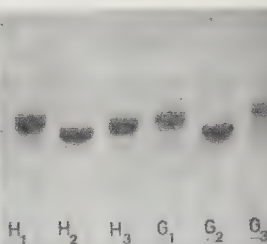


FIGURE 6: Starch gel electrophoresis in 8 M urea and formate buffer (pH 8.0) of human β_2 -microglobulin (H_1 - H_3) and the minor form of guinea pig β_2 -microglobulin (G_1 - G_3). (H_1 , G_1) Untreated protein; (H_2 , G_2) protein reduced and alkylated in the presence of 8 M urea; (H_3 , G_3) protein reduced and alkylated in the absence of urea. Electrophoresis proceeded in cathodal direction from the bottom to the top.

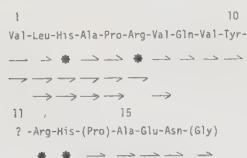


FIGURE 7: Amino acid sequence of residues 1 to 18 of the major form of guinea pig β_2 -microglobulin. (→, →, and *) denote sequence analysis of intact protein in the automatic sequencer with phenylthiohydantoins identified: (→) by thin-layer chromatography; (→) by hydrolysis to free amino acid followed by amino acid analysis; (*) by colorimetric test. (→) Indicates sequence determination of the protein by the dansyl-Edman technique. Residues in parentheses were detected on a single analysis and, therefore, are less certain.

creased by about the same extent after reduction and alkylation in the presence of urea. The minor component, reduced and alkylated in the absence of urea, showed a weak additional band with the same mobility as the minor component that was treated in the presence of urea. The comparisons of the minor component and human β_2 -microglobulin are illustrated in Figure 6. These observations suggest that the two guinea pig proteins and human β_2 -microglobulin have disulfide-linked loops of similar size, and that only the intrachain disulfide bridge of the minor guinea pig component is sensitive to reduction in the absence of a denaturing agent.

Evidence for Self-Association. As described above and illustrated in Figure 3, the major form of guinea pig β_2 -microglobulin in moderate amounts (5–15 mg) emerged from the Sephadex G-100 column (145 × 3 cm) as a symmetrical peak, whereas relatively large amounts (50–70 mg) of material appeared as asymmetric peaks with smaller elution volumes. This difference suggests that the protein self-associates at increasing concentration. Further evidence for self-association was obtained by analyses of the major β_2 -microglobulin species in the ultracentrifuge. The sedimentation coefficient showed a marked protein concentration dependence with an increasing coefficient at increasing concentration. The concentration dependence appeared linear in the range examined (0.7 to 8 mg/mL) and can be described by the equation $s_{20,w} = 1.605 S + 0.139c$, where c is the protein concentration in mg per mL. On sedimentation equilibrium analysis the plot of $\ln c$ vs. r^2 was curved with increasing slope toward the bottom of the cell.

Other Physicochemical Properties. Some physical proper-

TABLE III: Physicochemical Properties of the Major Form of Guinea Pig β_2 -Microglobulin.

Sedimentation coefficient, $s_{20,w}^0$	1.6 S
Stokes' molecular radius, r_s	1.6 nm
Partial specific volume, $\bar{V}$ (calcd) ^a	0.733 mL/g
Apparent diffusion coefficient, $D_{20,w}$	133 $\mu\text{m}^2/\text{s}$
Molecular weight	
From sedimentation and diffusion coefficients	11 000
By gel chromatography in 6 M Gdn-HCl	11 700
By electrophoresis in sodium dodecyl sulfate	11 500
From amino acid composition	11 544
Frictional ratio	1.12
Absorption coefficient at 280 nm (pH 7.0)	$1.52 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$
Isoelectric point at 5 °C	6.7
at 20 °C	6.6

^a From amino acid composition.

ties of the major form of guinea pig β_2 -microglobulin are presented in Table III. Physicochemical studies of the minor form were precluded by shortage of materials. All figures in Table III are average values from analyses of at least two different preparations. It is apparent that the molecular weights obtained by chromatography in 6 M guanidine hydrochloride, by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, and by calculations from the sedimentation and diffusion coefficients or from the amino acid composition, are in good agreement and indicate a value of about 11 500. The sedimentation equilibrium data were difficult to interpret because of the tendency of the protein to self-associate (see above). On polyacrylamide gel electrophoresis in sodium dodecyl sulfate, the major and the minor forms seemed to have the same molecular weight.

The light-absorption spectrum in the ultraviolet range was that of a typical protein. The absorption coefficient at 278 nm and pH 7.0 was estimated to be $1.53 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ with a value of 11 500 for the molecular weight. A similar absorption coefficient ($1.66 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) was obtained by calculation according to Wetlaufer (1962).

Isoelectric focusing experiments on two preparations gave single, symmetrical peaks with the pI value given in Table III.

Partial Amino Acid Sequence. The amino terminal sequence of the first 18 residues is shown in Figure 7, as well as the methods used for identification of each residue. In preliminary studies Pth-amino acids were detected only weakly at positions 6, 11, 12, and 14. Position 6 was tentatively identified as glutamic acid or glutamine (Cunningham et al., 1977) by the dansyl-Edman technique. Subsequent studies by us and by others (Wolfe & Cebra, 1978) have shown that residue 6 is arginine. We have also been able to provide a more definitive assignment of the arginyl residue at position 12. In all of our experiments, after position 18, identification was ambiguous, probably due to incomplete Edman degradation of the asparaginyl-glycine sequence.

Discussion

β_2 -Microglobulin is apparently eliminated from the blood mainly in the kidneys (Bernier & Conrad, 1969), and urine from patients with renal tubular disease has been the best source for the isolation of human β_2 -microglobulin (Berggård, 1965; Berggård & Bearn, 1968). We have previously used sodium chromate to induce renal damage and β_2 -microglob-

TABLE IV: Comparison of the Amino Acid Composition of Human, Rabbit, Mouse, and Cow, β_2 -Microglobulins with that of Guinea Pig β_2 -Microglobulin.^a

Amino acid	Guinea pig β_2 -microglobulin	Difference between			
		Human & guinea pig	Rabbit & guinea pig	Mouse & guinea pig	Cow & guinea pig
Asp	13	-1	+2	-3	-2
Thr	3	+2	+1	+4	-1
Ser	9	+1	-3	-2	-1
Glu	10	+1	+1	+1	+2
Pro	7	-2	+1	+1	+2
Gly	3	0	0	+1	0
Ala	4	-2	-2	+1	-3
$\frac{1}{2}$ -cystine	2	0	0	0	0
Val	9	-2	+1	-4	-4
Met	1	0	0	+3	-1
Ile	5	0	-2	+1	+1
Leu	7	0	0	-3	+1
Tyr	4	+2	+1	0	+2
Phe	5	0	0	-1	-1
Lys	8	0	0	+1	+1
His	5	-1	-1	-1	-1
Arg	3	+2	+1	+1	+2
Trp	2	0	0	0	0
Total	100	0	0	0	-3

^a Given as deviation from the composition of the major form of guinea pig β_2 -microglobulin. Data for the human protein are from Berggård & Bearn (1968), for the rabbit protein from Berggård (1974), for the mouse protein from Natori et al. (1975), and for the cow protein from Groves & Greenberg (1977).

ulinuria in rabbits (Berggård, 1974). In this investigation the same agent was chosen for treatment of guinea pigs and yielded two forms of the protein, differing in isoelectric point and amino acid composition (Table II).

The immunological cross-reactions between rabbit β_2 -microglobulin and the two proteins isolated from guinea pig urine indicated that the latter proteins are homologues of β_2 -microglobulin. This was established by the finding that the two forms have almost identical amino acid compositions (Table II) and by comparison of the partial amino acid sequence of the major form of the guinea pig protein with corresponding sequences of β_2 -microglobulin from other species (Figure 8). The sequence of residues 1-18 of the guinea pig protein resembles closely those of β_2 -microglobulins from all other species. As is seen among the other β_2 -microglobulins, most variations are seen in residues 1 to 7, whereas residues 8-18 are more conserved.

Comparisons of amino acid compositions of four β_2 -microglobulins suggest that the entire primary structures of β_2 -microglobulins from various species are similar (Table IV). The β_2 -microglobulins from all four animals contain two half-cystinyl residues that could form disulfide-loops similar to those characteristic of human β_2 -microglobulin and the immunoglobulin domains (Cunningham et al., 1973). The results of our experiments with starch gel electrophoresis in urea and the apparent absence of free sulfhydryl groups in guinea pig β_2 -microglobulin suggest that the guinea pig and human proteins do have analogous disulfide loops.

Guinea pig and human β_2 -microglobulins also have very similar physical-chemical properties. The molecular sizes, sedimentation coefficients, molecular weights, and frictional ratios are almost the same for the two proteins, and also for isolated immunoglobulin domains (Karlsson et al., 1972; Karlsson, 1974; Berggård, 1976). The low frictional ratios of these molecules (1.1 to 1.2) indicate that they have compact, globular shapes.

Like its human counterpart (Berggård & Bearn, 1968), guinea pig β_2 -microglobulin occurs in free form in serum and

	1	5	10	15	20
Human	Ile-Gln-Arg-Thr-Pro-Lys-Ile-Gln-Val-Tyr-Ser-Arg-His-Pro-Ala-Glu-Asn-Gly-Lys-Ser				
Rabbit	Val-Gln-Arg-Ala-Pro-Asn-Val-Gln-Val-Tyr-Ser-Arg-His-Pro-Ala-Glu-Asn-Gly-Lys-Asp				
Dog	Val-Gln-His-Pro-Lys-Ile-Gln-Val-Tyr-Ser-Arg-His-Pro-Ala-Glu-Asn-Gly-Lys-Pro				
Mouse	Ile-Gln-Lys-Thr-Pro-Gln-Ile-Gln-Val-Tyr-Ser-Arg-His-Pro-Pro-Glu-Asn-Gly-Lys-Pro				
Rat	Ile-Gln-Lys-Thr-Pro-Gln-Ile-Gln-Val-Tyr-Ser-Arg-His-Pro-Pro-Glu-Asn-Gly-Lys-Pro				
Cow	Ile-Gln-Arg-Pro-Lys-Ile-Gln-Val-Tyr(Ser)Arg-His-Pro-Pro-Glu(Asx)Gly-Lys-Pro				
Guinea pig	Val-Leu-His-Ala-Pro-Arg-Val-Gln-Val-Tyr- ? Arg-His(Pro)Ala-Glu-Asn(Gly) ? ?				

FIGURE 8: Comparison of the NH₂-terminal amino acid sequences of β_2 -microglobulins from different species. The data for the human protein are from Cunningham et al. (1973); for the rabbit protein from Cunningham & Berggård (1975); for the dog protein from Smithies & Poulik (1972b); for the mouse protein from Appella et al. (1976); for the rat protein from Uhr et al. (1977); and for the cow protein from Groves & Greenberg (1977) and Becker et al. (1977). Residues differing from the human are underlined.

urine and appears on cell surfaces. Schwartz et al. (1976) have demonstrated that GLPA histocompatibility antigens, solubilized from guinea pig lymphocytes, are 40'000 dalton glycoproteins that are noncovalently associated with a 12 000 dalton molecule. The latter molecule is guinea pig β_2 -microglobulin (Schwartz, B. D., Cigén, R., Berggård, I., and Shevach, E. M., in preparation). Solubilized guinea pig I region-associated (Ia) antigens do not contain any subunit of the size of β_2 -microglobulin (Schwartz et al., 1976). In agreement with these observations, we have found that β_2 -microglobulin is associated with GLPA antigens, but not with Ia antigens, on the surfaces of intact guinea pig lymphocytes (Björck et al., 1977). Analogous results have previously been obtained for the human HLA antigens, the murine H-2 antigens, and the human and murine Ia (or Ia-like) antigens, see Björck et al. (1977).

The histocompatibility antigens with which β_2 -microglobulin is associated on cell surfaces are characterized by their extensive genetic polymorphism (Klein, 1975). In contrast, no genetic variant of β_2 -microglobulin has been unambiguously identified in any species. Such variants could be particularly valuable because they would allow more definitive determi-

nation of the locations of β_2 -microglobulin genes in mammalian chromosomes (Goodfellow et al., 1975) and their possible relationship to the genes for histocompatibility antigens and immunoglobulins. Variant forms of the protein, such as degradation products or polymers of defined size, would also be valuable in that they might provide clues to the function of the protein and its interaction with histocompatibility antigens.

The two forms of guinea pig β_2 -microglobulin gave reactions of immunological identity on immunodiffusion analyses, but differed in charge and lysine content. As shown by ion-exchange chromatography and electrophoresis, the net charge of the minor form was more negative than that of the major form in the pH range of 8 to 9 consistent with difference in amino acid composition. Two forms of human β_2 -microglobulin have also been detected by their difference in isoelectric point (Hall et al., 1977; Berggård, unpublished observations). These two forms have not been isolated so the nature of the difference is not known. In both species, the minor form of the protein might be synthesized separately or be a degradation product of the major form. Definitive evidence for either of these hypotheses will depend on the isolation of larger amounts of the minor form for peptide mapping studies and amino acid sequence determination.

Some evidence that the minor form may arise by degradation of the major form in the guinea pig is suggested by the fact that the intrachain disulfide bond of the minor form is susceptible to reduction even in the absence of a denaturing agent and hence the minor form may be a modified form of the protein. In contrast, the disulfide bonds of the major form of the protein and of human β_2 -microglobulin could be reduced only after denaturation. Although the charge difference between the two β_2 -microglobulin species is consistent with our finding of 7 lysyl residues in preparations of the minor species vs. 8 in the major, we cannot rigorously exclude the possibility that the difference in lysyl residues is due to an impurity that is copurified with one or the other of the forms. If the difference in lysyl residues is due to an impurity, the minor β_2 -microglobulin species may be derived from the major species by deamidation of labile glutamyl or asparaginyl residues. Modification of proteins *in vivo* by loss of amide groups is a well-known phenomenon (Flatmark & Sletten, 1968).

The domains of different immunoglobulin chains occur in pairs (Edelman, 1970). As human β_2 -microglobulin is homologous to such domains (Peterson et al., 1972; Cunningham et al., 1973) and apparently is a subunit of the HLA histocompatibility antigens (Nakamura et al., 1973; Grey et al., 1973; Peterson et al., 1974), it may be bound to a similar domain in the larger subunit of these antigens. Some fragments corresponding to single domains of immunoglobulin chains appear in solution as monomers (Karlsson et al., 1972), whereas other such fragments appear as dimers (Epp et al., 1974). Human β_2 -microglobulin occurs in monomeric form at pH 8 (Berggård & Bearn, 1968). At the same pH, the major form of guinea pig β_2 -microglobulin was found to self-associate when its concentration was increased. This difference in the behavior of the human and guinea pig proteins could, at least partly, be due to the fact that the self-association was recorded at pH 8, which is closer to the isoelectric point of guinea pig protein (6.7) than to the isoelectric point of the human protein (5.7) (Hall et al., 1977). The self-association of the guinea pig protein might be promoted by the β_2 -microglobulin region that, on the cell surface, interacts with a region on the larger subunit of histocompatibility antigens.

A correlation may exist between the tendencies of a β_2 -microglobulin to self-associate and to crystallize. Bovine β_2 -microglobulin (Groves & Greenberg, 1977) was originally

reported as a crystalline, 43 000 dalton molecule (Groves et al., 1963) isolated from milk and colostrum. Studies of the molecule in dilute solution and in the crystal indicated that the predominant form was the monomer (Becker et al., 1977). The protein, however, did aggregate at higher concentrations, suggesting that the aggregation may reflect the ability of the molecule to crystallize.

Acknowledgment

We thank Ms. Boel Wieslander and Ms. Britt-Marie Palmblad for excellent technical assistance and Dr. Håkan Pertofto for performing the ultracentrifugations.

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STRUCTURAL DIFFERENCE BETWEEN THE TWO FORMS OF GUINEA-PIG
 β_2 -MICROGLOBULIN AND THEIR OCCURRENCE IN INBRED GUINEA-PIG STRAINS

Running title: C-terminal sequence analysis of guinea-pig
 β_2 -microglobulin forms.

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SUMMARY

The structural difference between two forms (basic and acidic) of guinea-pig β_2 -microglobulin (β_2m) has been established. Both forms are present in urine from inbred guinea-pig strains.

The β_2m forms were each digested with carboxypeptidases. Released amino acids were separated from remaining protein, dansylated and analysed by two dimensional thin layer chromatography on polyamide sheets.

Digestion with carboxypeptidase A, contaminated with carboxypeptidase B, released lysine from the basic form, but no detectable amino acid from the acidic β_2m . Asparagine was released after 30 min digestion of acidic β_2m by carboxypeptidase Y. In the basic digestion asparagine was not detected until after 2 h when also lysine and unidentified amino acids were released. Lysine was not found in the corresponding acidic β_2m digest which in other aspects was similar to that of basic β_2 . Acidic β_2m , with asparagine as C-terminal amino acid, is thus one amino acid (lysine) shorter than basic β_2m . SDS-polyacrylamide gel electrophoretic studies of the two forms also support this concept.

The presence of the two β_2m forms in urine from inbred guinea-pig strains 2 and 13, shown by gel filtration and ion exchange chromatography, makes it unlikely that the two forms are a result of genetic polymorphism.

INTRODUCTION

β_2m , the light chain of MHC Class I antigens, is a globular protein of 11.800 mol. wt. It is structurally related to domains of immunoglobulins and MHC Class I and II antigens (Smithies and Poulik, 1972; Orr *et al.*, 1979; Träggårdh *et al.*, 1979; Larhammar *et al.*, 1981).

Human β_2m was originally purified from urine from patients with tubular dysfunctions (Berggård and Bearn 1968). Since then β_2m has been isolated from a number of mammals (cf Berggård *et al.*, 1980) and some birds (Winkler and Sanders 1977; Lillehoj *et al.*, 1982). More recently β_2m -like "activity" has been detected in invertebrates (Shalev *et al.*, 1981).

Two forms with different amino acid composition and electrophoretic mobility were detected when the guinea-pig β_2m homologue was purified (Cig n *et al.*, 1978). Variants of β_2m with different net charges have also been found in β_2m from man (Hall *et al.*, 1977), rat (L gdberg *et al.*, 1979), mouse (Goding and Walker 1980; Michaelson *et al.*, 1980), and rabbit (Bj rck *et al.*, 1981). Except in mouse, where the β_2m variants are the result of strain-related polymorphism consisting of an amino acid substitution (Gates *et al.*, 1981), the structural background of the β_2m variants is not well known.

Although speculations regarding the structural difference between these two guinea-pig β_2m forms suggest the difference to be located in the C-terminal end of the protein (Cig n *et al.*, 1978; Wolfe and Cebra 1980) it has not yet been shown in these forms. In this study, the amino acids released from the two guinea-pig β_2m forms by carboxypeptidases, have been analysed. Also, the presence of the two forms in urine from inbred guinea-pig strains has been investigated.

MATERIALS AND METHODS

Proteins, urines and antiserum

Both forms (basic and acidic) of guinea-pig β_2m were purified from urine as has been described (Cig n *et al.*, 1978). Also, human β_2m was isolated from urine (Bergg rd and Bearn 1968). Urine from healthy inbred guinea-pigs, strain 2 and 13, was kindly provided, with 0.05% NaN_3 added, by Dr. Lennart L gdberg, National Institutes of Health, Bethesda, MD. Goat antiserum against the basic form of guinea-pig β_2m was prepared and analysed as previously reported (Bj rck *et al.*, 1977.).

Carboxypeptidase Y and PMSF-treated carboxypeptidase A were purchased from Worthington Biochemical Corp. (New Jersey). CPA, that, according to the manufacturer, contained small amounts of chymotrypsin and carboxypeptidase B, was treated with DFP prior to use.

Analysis of the C-terminal amino acids of the two guinea-pig β_2m forms.

All chemicals and reagents were of ultrapure or equivalent grade when available.

Reduction and alkylation. β_2 M (5 mg/ml) was dissolved in 1.0 M Tris-HCl, pH 8.5, with 6.0 M guanidine-hydrochloride. Reduction with 0.02 M dithioerythritol was carried out for 3 h and alkylation with 0.05 M iodoacetamide for 1 h in the dark at room temperature. The β_2 m forms were then dialysed exhaustively against distilled water and lyophilized.

Digestion with carboxypeptidases. The lyophilized β_2 m, 50-80 μ g (4.3-7 nmol)/digestion time, was dissolved at a concentration of 2.0 mg/ml in 0.2 M N'-ethylmorpholine acetate buffer, pH 8.0, with 0.1% SDS. Enzyme, 1.0 mg/ml in the same buffer, was added to a molar enzyme-protein ratio of about 1:20. CPA, suspended in 0.1 ml 1% NaHCO_3 , was dissolved by a few drops of 0.1 M NaOH as described (Ambler, 1972). Samples where β_2 m or the enzyme were left out were prepared as controls. The digestions were performed at 37^o C. Aliquots of 25-40 μ l were withdrawn at intervals specified in Results. The proteins were immediately precipitated with 10-20 μ l icecold 20% trichloro-acetic acid and spun down (3000 x g, 15 min). The supernatant was evaporated to dryness three times and washed with 100 μ l distilled water in between. Finally the solid material was redissolved in 35 μ l of 0.75 M NaHCO_3 , pH 9.8.

Dansylation. The pH of the redissolved samples was controlled by using indicator paper and a 2 μ l Hamilton syringe. Usually, 3-4 μ l 2 M NaOH had to be added to the samples. Twenty-five μ l of DNS-Cl (Aldrich-Europe, Beerse, Belgium) (5 mg/ml) in distilled acetone was added to each digest or control. After 40 min in 37^o C the dansylations were stopped by 6 μ l formic acid.

Two dimensional thin layer chromatography. One μ l of the dansylated samples was applied on polyamide layer sheets (6.5 x 6.5 cm). (Cheng-Chin Trading Co., Ltd., Taipei, Taiwan) according to Woods and Wang (1967). The following separation system was used: 1.5% formic acid (solvent 1); benzene-acetic acid, 9:1 v/v (solvent 2); ethylacetate-acetic acid-methanol, 20:1:1 v/v (solvent 3); 0.05 M Na_3PO_4 in 25% aqueous ethanol (solvent 4) (Narita *et al.*, 1975). The separation with solvent 1 was directed in right angle to those of solvents 2-4 which were run in the same direction. The dansylated amino acids were visualised by UV-light. To identify the unknown amino acids dansylated reference amino acids (BDH Chemicals Ltd., Poole, U.K.) were applied in the same spot as the

unknown sample. A standard sheet was then compared to sheets with unknowns only.

SDS-polyacrylamide gel electrophoresis.

SDS-PAGE was performed in slabs according to Neville (1971). The concentration of acrylamide in the separation gel was 13.7% and the cross-linking 3.7%.

Analysis of urine from inbred guinea-pig strains for the presence of two guinea-pig β_2m forms.

Protein solutions were concentrated by ultrafiltration using Visking dialysis tubing 24/32 in. (Union Carbide Corp., Chicago, IL) (Berggård, 1961).

Gel filtration and ion exchange chromatography were performed with Sephadex-G 100 and Sephadex DEAE-A50 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Total protein was determined by the absorbance at 280 nm, and β_2m concentrations were estimated by single radial immunodiffusion (Mancini *et al.*, 1965) or by radioimmunoassay. The Chloramine-T method of Greenwood *et al.*, (1963) was used to radiolabel β_2m with ^{125}I (Amersham, U.K.). Immune complexes were precipitated with the polyethylene glycol exclusion method (Plesner *et al.*, 1975).

RESULTS

Analysis of the C-terminal amino acid of the two forms of guinea-pig β_2m .

All β_2m preparations used were carefully controlled by SDS-PAGE for contaminations prior to digestion with carboxypeptidases.

Digestion with CPA. Initial digestion experiments were performed with CPA. In spite of repeated prolonged (over night) digestions no detectable amino acid was released from the acidic β_2m by CPA as judged from two dimensional thin layer chromatography on polyamide sheets.

In parallel experiments the basic form was also subjected to digestion, initially merely as a control. Unexpectedly one spot, beyond control levels, was seen weakly after 30 min of digestion, but increased after

longer incubation periods (Fig. 1). The spot was visible after the first two developments but appeared more clearly after separation with the solvents 3 and 4. The identity of the spot was tested against standard amino

DIGESTION- TIME (min)	GUINEA-PIG β_2 -MICROGLOBULIN		
	BASIC		ACIDIC
	CPA/B	CPY	CPY
0	—	—	—
15	—	—	(□)
30	■	—	□
60	■	N.D.	N.D.
120	■	■ □ ● ○	□ ● ○

Fig. 1. Digestion of basic and acidic guinea-pig β_{2m} with carboxypeptidase A contaminated with carboxypeptidase B (CPA/B) and with carboxypeptidase Y (CPY). The digests were separated in two directions by solvents 1, 2 and 3 on polyamide sheets. (■) lysine; (□) asparagine; (●, ○) unidentified amino acids; N.D. not determined.

acids and established as lysine. Several different preparations of β_{2m} were investigated with the same result. Digestion of native protein was, however, not successful.

Digestion with CPY. As no C-terminal amino acid of the acidic β_{2m} was possible to determine with CPA, digestions with CPY were undertaken.

After digestion for 2 h with CPY and separation with solvents 2 and 3, three spots in the acidic and four in the basic digest were seen in addition to the background. Probably more amino acids were released but could not be separated from the background which was rather high close

to the front of the second development. The fluorescence in this area was more intense in the β_2m digests than in controls of enzyme only. No differences between the two forms could be detected in this area. Fig. 2 shows the result of the 2-hour digestions after separation also with the third solvent. Here the background has disappeared.

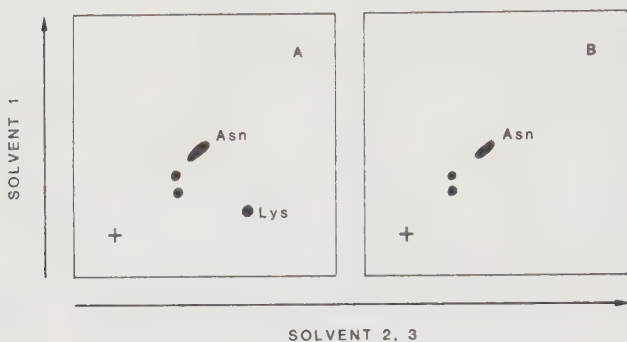


Fig. 2. Two dimensional thin layer chromatography on polyamide sheets of dansylated amino acids released from the basic (A) and the acidic (B) form of guinea-pig β_2m digested for 2 hours with carboxypeptidase Y. The sheets were developed with three solvents.

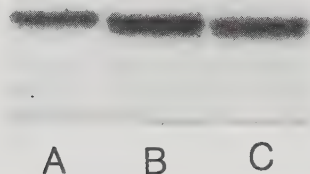
The fourth spot, only recognised in the basic β_2m digest, migrated as the amino acid detected in the CPA digest and was also identified as lysine. No released amino acids were seen in the 15 or 30 min digests of basic β_2m . Fluorescence close to the front of the second development was of the same intensity as controls of enzyme only.

One amino acid, also seen in the 2-hour digestion of both β_2m forms, was detected in the acidic β_2m digest of 30 min. Upon careful reinvestigation the spot could be seen weakly also after 15 min. The amino acid was identified as asparagine although it was difficult to separate it from aspartate. Again, the fluorescence in the vicinity of the front of the second separation was that of the background.

The other two spots were not further investigated. In digestions for longer periods than 2 h, lysine was also found in the digest of the acidic β_2m .

The possibility of different molecular weights in the two guinea-pig β_2m forms was analysed by SDS-PAGE. Although small, differences in mobility on SDS-PAGE are clearly visible in Fig. 3 indicating that the acidic β_2m is smaller than the basic form. Human β_2m is shown as a reference.

Fig. 3. SDS-polyacrylamide gel electrophoresis of human β_2m (A); guinea-pig β_2m , basic form (B); and guinea-pig β_2m , acidic form (C).



Analysis of the presence of two guinea-pig β_2m forms in urine from inbred guinea-pigs.

Normal urine from guinea-pig strain 2 (47 ml) and strain 13 (63 ml) was concentrated separately to 2.6 and 3.6 ml respectively and subjected to gelfiltration on Sephadex G-100 (1.3 x 105 cm) equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN₃. Fractions of 1.9 ml at a flow rate of 1.9 ml/h were collected. Absorbance at 280 nm was determined. The profile of total protein was essentially as earlier described (Cig n *et al.*, 1978). The major part of β_2m , detected by single radial immunodiffusion, was eluted at an approximate K_{av} of 0.6 and was also recognised as a small peak by the absorbance. These fractions were taken for further analysis. Small amounts of β_2m , detected by radio-immunoassay, were also found close to the void volumes.

The gelfiltered $\beta_2\text{m}$ -containing material was concentrated, dialysed against 0.04 M Tris-HCl, pH 8.0 and applied on columns of Sephadex DEAE-A50. Experimental details are given in the legend to Fig. 4 that shows the chromatogram of urine from guinea-pig strain 2. $\beta_2\text{m}$ was eluted in two

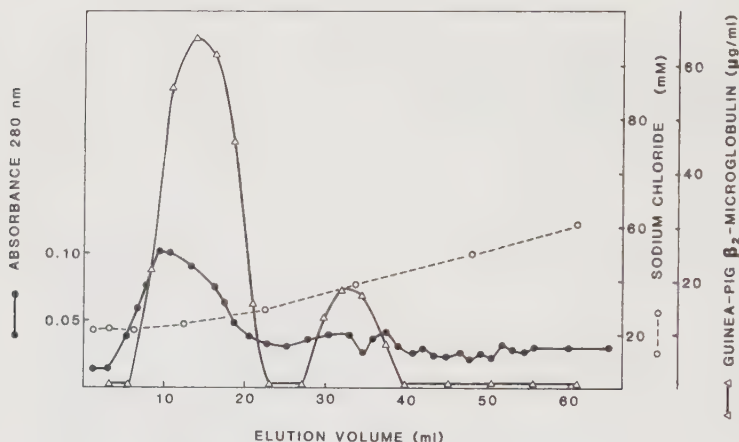


Fig. 4. Ionexchange chromatography of the $\beta_2\text{m}$ -containing pool from gelfiltration of normal urine from a strain 2 guinea-pig. The sample (2 ml) was dialysed against 0.04 M Tris-HCl, pH 8.0 and then subjected to a column (0.6 x 6.5 cm) of Sephadex DEAE-A50, equilibrated with the same buffer. The proteins were eluted with a linear gradient of NaCl (0-0.1 M) (○---○) and determined by the absorbance at 280 nm (●—●). Guinea-pig $\beta_2\text{m}$ (△—△) was measured by single radial immunodiffusion.

peaks at the same approximate sodium chloride concentrations as have been reported for pooled guinea-pig serum or urine from outbred guinea-pigs (Cig n, 1983). Corresponding ionexchange chromatography of material from guinea-pig strain 13 gave similar results.

DISCUSSION

The structural differences between the two guinea-pig $\beta_2\text{m}$ forms have been an object for discussion during recent years. However, the localisation of the proposed sequence difference in these two guinea-pig $\beta_2\text{m}$ forms has yet never been shown. The information available, concerning the structure of guinea-pig $\beta_2\text{m}$ (Cig n *et al.*, 1978; Wolfe and Cebra, 1980) has

suggested that the structural difference is situated at the C-terminal end of the protein. Accordingly, a strategy based on digestions with carboxypeptidases was chosen. As Wolfe and Cebra (1980) reported digestion with CPY, on presumably a mixture on both forms unsuccessful, CPA was tried initially.

Much to my surprise lysine was released from the basic form and nothing from the acidic. The release of lysine is explained by the contamination of CPA with CPB reported by the manufacturer. The enzyme was also contaminated with chymotrypsin, but as no other spots were visible, the DFP treatment must have been effective. A multitude of amino acids was recorded in one experiment when no DFP was added.

Digestion with CPY released asparagine, although the separation from aspartate was subtle, as the first amino acid in the acidic β_{2m} digest and lysine in the basic digest. Lysine was not seen in the acidic digest but after the appearance of lysine, asparagine was detected in the basic β_{2m} digest.

Thus, it is clearly shown that these forms of guinea-pig β_{2m} have different C-terminal amino acids (Fig.5), and the results are in agreement with the sequence of guinea-pig β_{2m} reported by Wolfe and Cebra (1980).

GUINEA-PIG			
β_{2m}	90	95	99
BASIC	...Pro-Lys-Ile-Val-Lys-Trp-Asp-Pro-Asn-Lys		
ACIDIC	...Pro-Lys-Ile-Val-Lys-Trp-Asp-Pro-Asn		

Fig. 5. C-terminal amino acid sequence of basic guinea-pig β_{2m} from Wolfe and Cebra, (1980) and the proposed C-terminal sequence of acidic guinea-pig β_{2m} .

The molecular weights based on sequence analyses are 11.728 for human β_{2m} (Cunningham *et al.*, 1973; Parker and Strominger, 1982) and 11.415 for guinea-pig β_{2m} (Wolfe and Cebra, 1980). The lack of the C-terminal lysine

in the acidic β_2m would then result in a molecular weight of 11.287.

Indeed the mobility analysis on SDS-PAGE also supports the fact that the acidic β_2m is shorter than the basic form as well as the amino acid composition previously described (Cig n *et al.*, 1978).

Once it was argued that the two forms might represent allelic variants of β_2m , that more recently have been found in the mouse system (Goding and Walker, 1980; Michaelson *et al.*, 1980; Gates *et al.*, 1981). However, in distribution studies of the two guinea-pig β_2m forms no acidic β_2m was found on membranes from liver tissue from outbred animals, but both forms were detectable in serum from such animals (Cig n, 1983). This was interpreted as arguments against the possibility that the two forms not were results of genetic polymorphism. Further support to this concept is given in this paper. Ionexchange chromatographies of urine from inbred guinea-pig strains 2 and 13 clearly show two distinct β_2m peaks. The chromatographies are very similar to those earlier presented on serum and urine from outbred guinea-pigs (Cig n, 1983).

The origin of the two forms of guinea-pig β_2m is still unknown. It would be easy to believe that the C-terminal lysine in the basic form randomly is cleaved off in the blood by an enzyme with carboxypeptidase activity. That could also explain why only the basic form is found on membranes (Cig n, 1983). On the other hand, it is hard to see how this could be accomplished as digestion with carboxypeptidases of native protein was unsuccessful. The acidic β_2m must, after the digestion *in vivo*, regain its tertiary structure as it reacts well with antibodies. However, more confirmative conclusions await more detailed studies of the three-dimensional structure of guinea-pig β_2m and the orientation of its C-terminal amino acid.

Of particular interest is also whether the C-terminal amino acid is crucial for any of the more functional aspects of β_2m - i.e. its interaction with MHC Class I antigens - or not. Such an implication may have been given by the absence of the acidic guinea-pig β_2m on membranes (Cig n, 1983).

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Faculty, University of Lund, Sweden, and the Royal Physiographic Society, University of Lund, Sweden.

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Relationships Between β_2 -Microglobulin and Alloantigens Coded for by the Major Histocompatibility Complexes of the Rabbit and the Guinea Pig

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Treatment of rabbit and guinea pig lymphocytes with Fab' fragments of anti- β_2 -microglobulin completely inhibited the cytotoxic effects of alloantisera to RLA or GPLA antigens, respectively. Aggregation of β_2 -microglobulin on the lymphocyte surface by successive incubations with goat anti- β_2 -microglobulin and F(ab')₂ fragments of rabbit anti-goat IgG also made rabbit lymphocytes resistant to lysis by anti-RLA, and guinea pig lymphocytes resistant to lysis by anti-GPLA. The two kinds of pretreatment of guinea pig lymphocytes did not affect the cytotoxicity of antisera directed against guinea pig Ia antigens. These results in conjunction with previous findings in the mouse and in man suggest that β_2 -microglobulin on the lymphocyte surface in mammals is generally associated with major serologically defined histocompatibility antigens but not with I-region-associated antigens.

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The major serologically defined histocompatibility antigens and the I-region-associated (Ia) antigens are (at least in higher vertebrates) determined by genes present in the major histocompatibility complex (35). Previous work on major histocompatibility antigens released from cell surfaces has shown that β_2 -microglobulin (mol.wt., 12,000) is linked to the HLA antigens in man (15, 25, 29), to the H-2 antigens in the mouse (26, 31, 34, 41), and apparently also to the Ag-B antigens in the rat (18). It has been demonstrated that β_2 -microglobulin is associated with HLA and H-2 antigens not only after solubilization of these antigens but also at the cell surface (20, 27, 30, 31, 36). In the mouse, cell surface Ia antigens apparently lack a β_2 -microglobulin subunit (10, 14, 40). This also seems to be

true for Ia-like antigens in man (9, 17). But such a subunit has been found in a murine cell-cell interaction factor that possesses Ia antigenic determinants, and Ia antigens isolated from mouse serum have been reported to contain β_2 -microglobulin (1, 8).

In the present work, we have examined two more animals, the rabbit and the guinea pig, with regard to association of β_2 -microglobulin with alloantigens determined by their major histocompatibility complexes. This study and the previous investigations of human and murine alloantigens (20, 27, 30, 31, 36) suggest that β_2 -microglobulin on intact mammalian lymphocytes is generally associated with the major serologically defined histocompatibility antigens (RLA antigens in the rabbit and GPLA antigens in the guinea pig) but not

with the I-region-associated antigens. According to previous work on guinea pigs, a 12,000-dalton protein occurs bound to solubilized GPLA antigens but not to solubilized Ia antigens (12, 33). Our finding of a GPLA- β_2 -microglobulin association in intact lymphocytes indicates that the 12,000-dalton protein of solubilized GPLA antigens is β_2 -microglobulin.

MATERIALS AND METHODS

Lymphocytes. Lymphocytes from outbred rabbits and guinea pigs were purified from defibrinated blood. After sedimentation for 30 min at 37°C the supernatant was applied to a cotton wool column (19). The effluent obtained from the column was subjected to gradient centrifugation (7) on Lymphoprep, density 1.077 (Nyegaard & Co. A/S, Norway).

β_2 -Microglobulins. The purification of rabbit and guinea pig β_2 -microglobulin from urine of animals treated with sodium chromate has been described (2, 3). Further details of purification and chemical properties will be published later.

Antisera. Antisera against rabbit and guinea pig β_2 -microglobulin were raised in two goats. A mixture of equal volumes of protein solutions (1 mg/ml) and complete Freund's adjuvant (Difco, USA) was inoculated intracutaneously in several sites in the back and intramuscularly in all four legs. Each animal was given 0.4 mg rabbit or guinea pig β_2 -microglobulin. The immunization was repeated 1 month later, and the animals were bled after another 10 days. Incomplete Freund's adjuvant (Difco) was used for the second immunization. The two antisera gave only a single precipitin arc when tested on immunoelectrophoresis against various amounts of concentrated urinary proteins from rabbits or guinea pigs treated with sodium chromate.

Antisera against normal goat IgG (see below) were prepared in two rabbits. The immunization procedure has been described (4).

Alloantisera to RLA antigens 1 and 2 were generous gifts from Dr. H. Matej. These reagents, raised by allogeneic skin graftings, have previously been described by Dr. Matej (22).

Dr. A. L. de Weck kindly supplied us with alloantisera to GPLA antigens B1 and B2 (13, 32) and to guinea pig Ia antigens 1.3 and 3 (13).

Preparation of IgG, F(ab')₂ fragments, and Fab' fragments. Purified IgG from a goat anti-rabbit β_2 -microglobulin antiserum, a goat anti-guinea pig β_2 -microglobulin antiserum, a pooled non-immune goat serum, and a rabbit anti-goat IgG antiserum was obtained by chromatography on DEAE-cellulose (Serva, Germany). A column, 4 × 19 cm, of the ion exchanger was equilibrated with 0.015M Tris-HCl buffer, pH 7.8. Serum dialyzed against the same buffer was applied to the column and eluted at pH 7.8 with a 4000-ml linear gradient of NaCl from 0 to 0.2M. Protein eluted up to 0.06M NaCl was concentrated by ultrafiltration and analyzed by agarose gel electrophoresis (16). Only one band in the gamma-globulin region was observed. F(ab')₂ fragments from the IgG preparations were obtained by pepsin digestion (28) followed by chromatography on Sephadex G-200 (Pharmacia, Sweden). Fab' fragments were prepared by reduction and alkylation of the F(ab')₂ fragments, again followed by chromatography on Sephadex G-200 (39).

Lymphocytotoxicity testing. A two-step microcytotoxicity test was used (37). One microliter of antiserum and 1 μ l of cell suspension (2000 cells) were incubated under liquid paraffin (ACO, Sweden) on microculture plates (Møller-Coates, Norway) for 30 min at 20°C. Five microliters of rabbit and guinea pig serum in a 2:1 proportion were added as complement source. Incubation with complement proceeded for 1 h at 20°C. The plates were read with an inverted microscope after addition of 2 μ l of 1% trypan blue in saline. The reactions were scored as follows: 0%–10% dead cells: –; 10%–20%: +; 20%–50%: ++; 50%–75%: +++; and 75%–100%: ++++.

Inhibition tests were performed by adding β_2 -microglobulin to the anti- β_2 -microglobulin antisera and by pretreating lymphocytes with Fab' fragments. In the latter case, lymphocytes were pretreated with Fab' fragments by two different techniques. First, 0.5 ml of lymphocytes in phosphate-buffered saline (PBS), pH

Table I. Effects of Fab' fragments of anti-rabbit β_2 -microglobulin on the lymphocytotoxicity induced by anti-RLA and anti- β_2 -microglobulin antisera

Sera	Rabbit 1 lymphocytes*		Rabbit 2 lymphocytes*	
	Untreated	Fab'-treated	Untreated	Fab'-treated
Anti-RLA 1	++	-	-	-
Anti-RLA 2	+++	-	++	-
Anti-rabbit β_2 -microglobulin	++++	-	++++	-
Non-immune rabbit or goat sera	-	-	-	-

* - = 0%-10% lysis, + = 10%-20% lysis, ++ = 20%-50% lysis, +++ = 50%-75% lysis, ++++ = 75%-100% lysis.

7.4, at a concentration of $1 \cdot 10^6$ cells/ml were incubated with an equal volume of Fab' fragments at a concentration of 3.0 mg/ml for 1 h at 4°C. The lymphocytes were washed four times in ice-cold PBS, and the cell concentration was adjusted to $2 \cdot 10^6$ cells/ml. Second, 1 μ l of Fab' fragments at a concentration of 3.0 mg/ml and 1 μ l of lymphocyte suspension (2000 cells) were incubated under liquid paraffin on microculture plates for 1 h at 20°C.

These two methods gave the same results when the pretreated lymphocytes were submitted to microcytotoxicity testing as described above. The second technique has the advantage of demanding less reagent, time, and labor.

Aggregation of cell surface β_2 -microglobulin. The technique used, often called the lysostrip method, has been described previously (5). Lymphocytes were first incubated with goat anti- β_2 -microglobulin antiserum (heat-inactivated and dialyzed against PBS, pH 7.4) for 2 h at room temperature and washed three times with ice-cold PBS. 0.3 ml of cells ($4 \cdot 10^6$ /ml) and 0.3 ml of rabbit anti-goat IgG F(ab') fragments (15 mg/ml) were allowed to react for 30 min at 0°C. Cells were washed three times with ice-cold PBS, and the concentration was adjusted to $2 \cdot 10^6$ /ml in RPMI-1640 (Flow Laboratories, U.K.). After another 30 min of incubation at 37°C the lymphocytes were quickly cooled to 0°C and 1M NaN₃ in PBS was added to the cells, giving the suspension a NaN₃ concentration of 10mM. Finally, the lymphocytes were subjected to cytotoxicity testing as previously described.

RESULTS

The relationship between β_2 -microglobulin and RLA antigens

Lymphocytes from eight rabbits were tested against a goat anti-rabbit β_2 -microglobulin antiserum. The antiserum was clearly cytotoxic to lymphocytes from all rabbits examined. The specificity of the reaction was studied by adding purified rabbit β_2 -microglobulin to the goat antiserum. This preincubation completely abolished the cytotoxicity of the reagent. Pretreatment of the lymphocytes with Fab' fragments against rabbit β_2 -microglobulin had the same effect (Table I), whereas Fab' fragments prepared from non-immune goat IgG did not influence the cytotoxic property of the antiserum.

By using anti-RLA antisera it was possible to classify the eight rabbits into RLA 1,2, RLA 2, and RLA 0 animals. Lymphocytes from rabbits of different specificities were pretreated with goat Fab' fragments against rabbit β_2 -microglobulin. As shown in Table I, this made the cells resistant to lysis by anti-RLA. Fab' fragments from non-immune goat IgG were used as a control. In this case no inhibition of anti-RLA lymphocytotoxicity was observed.

Further evidence of an association between β_2 -microglobulin and RLA was obtained by using the lysostrip technique. Aggregation of β_2 -microglobulin on the lymphocyte surface by successive incubations with goat anti-rabbit β_2 -microglobulin antiserum and F(ab')₂ fragments of rabbit anti-goat IgG effectively protected the cells against lysis by anti-RLA (Table II).

Table II. Inhibition of anti-RLA- and anti- β_2 -microglobulin-induced cytotoxicity on rabbit lymphocytes by redistribution of β_2 -microglobulin on the cell surface

Sera	Rabbit 3 lymphocytes*		Rabbit 4 lymphocytes*	
	Untreated	Pretreated†	Untreated	Pretreated†
Anti-RLA 1	++	-	-	-
Anti-RLA 2	++	-	+++	-
Anti-rabbit β_2 -microglobulin	++++	-	++++	-
Non-immune rabbit or goat sera	-	-	-	-

* See Table I for explanation of reaction scores.

† β_2 -microglobulin on the cells was redistributed by successive incubations with goat anti-rabbit β_2 -microglobulin antiserum and F(ab')₂ fragments of rabbit anti-goat IgG.

Association of β_2 -microglobulin with GPLA and guinea pig Ia antigens

The experimental procedures described above were used in experiments with lymphocytes from 32 guinea pigs. Specificity tests included absorption of the goat anti-guinea pig β_2 -microglobulin antiserum with purified guinea pig β_2 -microglobulin and inhibition on the lymphocyte surface with Fab' fragments of anti- β_2 -microglobulin IgG. In both cases, cytotoxicity was brought down to background levels. On the other hand, non-immune goat Fab' fragments did not affect the reactivity of the anti- β_2 -microglobulin antiserum, the anti-GPLA antisera, or the anti-Ia antisera.

Guinea pig lymphocytes pretreated with Fab' fragments against β_2 -microglobulin or manip-

ulated according to the lysostrip technique became resistant to anti-GPLA cytotoxicity, whereas the capability of anti-Ia to cause lympholysis remained unaffected. The results, summarized in Tables III and IV, indicate an association between GPLA and β_2 -microglobulin on the cell surface, whereas guinea pig Ia antigens do not seem to be bound to β_2 -microglobulin.

DISCUSSION

Previous work has shown that β_2 -microglobulin is part of the human HLA antigens (15, 20, 25, 27, 29, 30, 36), the murine H-2 antigens (26, 31, 34, 41), and probably also the rat Ag-B antigens (18), whereas murine mem-

Table III. Effects of Fab' fragments of anti-guinea pig β_2 -microglobulin on the lymphocytotoxicity induced by anti-GPLA, anti-Ia, and anti- β_2 -microglobulin antisera

Sera	Lymphocytes* from					
	Guinea pig 1		Guinea pig 2		Guinea pig 3	
	Untreated	Fab'-treated	Untreated	Fab'-treated	Untreated	Fab'-treated
Anti-GPLA B.1	+++	-	++	-	-	-
Anti-GPLA B.2	-	-	-	-	++	-
Anti-Ia 1.3	++	++	++	++	-	-
Anti-Ia 3	++	++	-	-	-	-
Anti-guinea pig β_2 -microglobulin	++++	-	++++	-	++++	-
Non-immune guinea pig or goat sera	-	-	-	-	-	-

* See Table I for explanation of reaction scores.

Table IV. Inhibition of anti-GPLA-, anti-Ia-, and anti- β_2 -microglobulin-induced cytotoxicity on guinea pig lymphocytes by redistribution of β_2 -microglobulin on the cell surface

Sera	Lymphocytes* from					
	Guinea pig 4		Guinea pig 5		Guinea pig 6	
	Untreated	Pre-treated†	Untreated	Pre-treated†	Untreated	Pre-treated†
Anti-GPLA B.1	+++	-	-	-	-	-
Anti-GPLA B.2	-	-	+++	-	+++	-
Anti-Ia 1.3	-	-	+	+	++	++
Anti-Ia 3	-	-	-	-	++	++
Anti-guinea pig β_2 -microglobulin	++++	-	++++	-	++++	-
Non-immune guinea pig or goat sera	-	-	-	-	-	-

* See Table I for explanation of reaction scores.

† β_2 -microglobulin on the cells was redistributed by successive incubations with goat anti-guinea pig β_2 -microglobulin antiserum and F(ab')₂ fragments of rabbit anti-goat IgG.

brane-bound Ia antigens and human Ia-like antigens seem to lack this subunit (9, 10, 14, 17, 40). We have investigated the possible association between β_2 -microglobulin and the major serologically defined histocompatibility antigens in two additional mammalian species, the rabbit and the guinea pig. The possible relationship between β_2 -microglobulin and guinea pig Ia antigens has also been examined.

The major histocompatibility system in the rabbit has been studied by several investigators (6, 11, 21, 22, 38). There have been considerable difficulties in finding an effective genetic approach to this histocompatibility system, mainly because of the lack of homozygous rabbit strains. Despite this, alloantisera have been raised in rabbits which detect alloantigens that most likely represent the rabbit counterpart of the murine H-2 or the human HLA system. The antisera used in our experiments detect the RLA 1 and RLA 2 antigens, which are well-defined antigens with relatively broad specificities. They are apparently determined by genes present at two different but closely linked loci (H. Matej, personal communication). The tissue distribution of RLA antigens (21, 23) and the fact that compatibility of these antigens prolongs the survival of skin allografts in related rabbits (24) are in analogy with the HLA and H-2 antigens. So far, no chemical

data are available concerning the structure of the RLA antigens. The results of this study indicate that the RLA antigens have a β_2 -microglobulin subunit and a basic structure similar to the structure of the H-2 and the HLA antigens.

The GPLA antigens were originally discovered by using alloantisera produced by cross-immunization of outbred guinea pigs (32). The structural characteristics of these antigens have been the subject of recently published studies (12, 33). Solubilized GPLA molecules were found to be composed of two subunits. The antigenic determinants were located to a 40,000-dalton glycoprotein. Non-covalently associated with this larger part of the molecule was a 12,000-dalton protein component. These data are in close agreement with studies concerning the subunit structure of solubilized H-2 and HLA antigens, where the 12,000-dalton component has been proved to be β_2 -microglobulin. In this paper we present strong evidence of a linkage between GPLA antigens and β_2 -microglobulin on intact cells. Our findings and the observation that one of our anti- β_2 -microglobulin sera precipitates both a 11,000- and a 40,000-dalton membrane protein (E. M. Shevach, personal communication) seem to establish that GPLA antigens are associated with β_2 -microglobulin.

Our present knowledge on the genetics and the structural characteristics of Ia antigens in the guinea pig has developed rapidly during the last 2 years (12, 13, 33). It has been reported that guinea pig Ia antigens, solubilized from cell surfaces, do not contain any subunit of β_2 -microglobulin size (12, 33). In agreement with this observation, we did not find any evidence of an association between Ia antigens and β_2 -microglobulin on intact cells, since blocking or aggregation of cell surface β_2 -microglobulin did not affect the activity of anti-Ia antisera. Previous studies have shown that murine cell surface Ia antigens apparently also lack a β_2 -microglobulin subunit (10, 14, 40). But Ia antigens isolated from serum (8) and culture supernatants (1) have been reported to contain β_2 -microglobulin. There is no obvious explanation of this discrepancy in the structure of cell-bound and free Ia antigens. One could, however, speculate about the possibility that a subpopulation of cell surface Ia antigens, carrying β_2 -microglobulin, is not detectable by present methods. Ia antigens associated with β_2 -microglobulin could also, hypothetically, represent molecules derived from secreting cells. Finally, secreted and/or shed Ia antigens might change their conformations in a way that favors an extracellular association with β_2 -microglobulin.

Previously published data on the structure of HLA and H-2 antigens, in combination with the results presented in this study and in the work of Schwartz et al. (33), make it reasonable to suggest that, in mammals, the major serologically defined histocompatibility antigens are generally associated with β_2 -microglobulin, whereas at least some cell-bound Ia antigens do not have a β_2 -microglobulin subunit.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. H. Matej and Dr. A. L. de Weck for generous gifts of allo-antisera. The excellent technical assistance of Ms. Solveig Månsson and Ms. Boel Wieslander is gratefully acknowledged. This investigation was supported by grants from the Swedish

Medical Research Council (Project 512) and the Medical Faculty, University of Lund.

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Received 23 March 1977

DISTRIBUTION OF THE TWO FORMS OF GUINEA-PIG β_2 -MICROGLOBULIN ORIGINALLY DETECTED IN URINE

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(First received 8 March 1983; accepted in revised form 8 July 1983)

Abstract—Two distinct forms of β_2 -microglobulin (β_2m) have been detected in pooled guinea-pig serum from outbred animals using ultrafiltration, gel filtration and ion exchange chromatography. These two forms probably correspond to the two β_2m forms, with different electrophoretic mobility, that have been previously identified in guinea-pig urine. In solubilized membranes from perfused guinea-pig livers only the basic form of β_2m could be found. These results may indicate that the two β_2m forms should not be ascribed to genetic polymorphism but rather are a result of post-translational modification.

INTRODUCTION

β_2m * has gained much interest during recent years. This is mainly because of: (1) its association with the heavy chain of the MHC Class I antigens (Nakamuro *et al.*, 1973; Peterson *et al.*, 1974) and other plasma membrane molecules (Vitetta *et al.*, 1975; Michaelson *et al.*, 1977; Fellous *et al.*, 1978; Stanton and Hood, 1980), (2) the structural homology to domains of immunoglobulins and MHC Class I antigens [a common evolutionary origin has been suggested (Smithies and Poulik, 1972; Orr *et al.*, 1979; Trägårdh *et al.*, 1979)], (3) the effects of antibodies against β_2m on lymphocyte activation and other immunological cell interactions [reviewed in Berggård *et al.* (1980) and Lögdberg (1982)], and (4) the use of β_2m as a marker of renal insufficiency and maybe also of certain malignancies and inflammatory diseases [for review see *Pathologie Biologie* (1978) and Karlsson *et al.* (1980)].

β_2m is a polypeptide with a mol. wt of 11,800 and was originally isolated by Berggård and Bearn (1968) from urine of patients with tubular dysfunctions. Since then β_2m homologues have been isolated from various biological fluids and tissues in a number of mammals [see Berggård *et al.* (1980)]. Non-mammalian β_2m has been purified from the chicken (Winkler and Sanders, 1977) and turkey (Lillehoj *et al.*, 1982).

During the isolation of the guinea-pig homologue from urine of animals with sodium chromate induced proteinuria two forms of the protein were detected (Cigén *et al.*, 1978). These differed in electrophoretic mobility and amino acid composition. It was argued that these forms may reflect a genetic polymorphism

or that one of the forms may originate from the other. Allelic variants of β_2m , due to an amino acid substitution, have been reported in certain mouse strains (Goding and Walker, 1980; Michaelson *et al.*, 1980; Gates *et al.*, 1981).

In this study the distribution of the two guinea-pig β_2m forms in serum and on liver cell membranes is analysed to exclude the possibility that one of the forms is a urinary degradation product.

MATERIALS AND METHODS

Animals

Healthy outbred guinea-pigs (0.6–1.2 kg) of either sex from local dealers were used.

Sera and proteins

Serum from guinea-pigs was obtained by heart puncture. Ten–twenty millilitres serum was recovered per animal and stored at -80°C until used. Normal goat serum was obtained by bleeding from the carotid arteries. Bovine serum for the radio-immunoassay was purchased from a local slaughter house. Goat antiserum against guinea-pig β_2m and rabbit antiserum against goat IgG were prepared and tested as described previously (Björck *et al.*, 1977). Guinea-pig β_2m was purified from urine as reported (Cigén *et al.*, 1978).

Other materials

The non-ionic detergent $\text{C}_{12}\text{H}_{25}\text{N}_4\text{O}_4$ (OCH₂CH₂)₃OH, abbreviated to C₁₂E₃ (commercial name Berol 0.58) [from Berol Kemi AB (Stenungssund, Sweden)], was a generous gift from Dr Gudrun Fredrikson, University of Lund, Sweden. Before use it was deionized on a mixed ion exchange resin (Fredrikson *et al.*, 1981). Sephadex G-100, Sephadex G-200 superfine and Sephadex-DEAE A-50 were from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were reagent grade or best quality available.

*Abbreviations: β_2m , β_2 -microglobulin; BSA, bovine serum albumin; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

Ultrafiltration

Visking dialysis tubing (Union Carbide Corp. Chicago, IL) were used for ultrafiltration (Berggård, 1961). Protein solutions were concentrated by using $\frac{23}{32}$ in. tubing. To separate serum proteins into one high and one low mol. wt fraction $\frac{8}{32}$ in. tubing was employed. This tubing has an approximate mol. wt cut-off at 30,000–40,000 under the conditions used.

Radiolabeling

Radiolabeling of protein with ^{125}I (Amersham, U.K.) was performed by the chloramine-T method (Greenwood *et al.*, 1963). Four micrograms of purified guinea-pig $\beta_2\text{m}$ was labeled with 2 mCi for use in the radioimmunoassay. For immunoprecipitation studies $\frac{1}{10}$ or $\frac{1}{5}$ respectively of the combined and conc. fractions of the gel chromatographies of the guinea-pig serum or the solubilized liver membranes were labeled with 1 mCi ^{125}I .

Protein determination

Total protein was measured by reading the absorbance at 280 nm or by the method of Folin, modified according to Lowry *et al.* (1951). BSA was used as the reference protein. Guinea-pig $\beta_2\text{m}$ at a concn of more than 5 mg/l was determined by single radial immunodiffusion (Mancini *et al.*, 1965). At lower concns a radioimmunoassay was used. Immune complexes were precipitated by the polyethylene exclusion method (Plesner *et al.*, 1975). The references were prepared in appropriate buffers and the detection limit was set to 10% inhibition, corresponding to 0.5 $\mu\text{g/l}$ $\beta_2\text{m}$.

Preparation of solubilized guinea-pig liver membranes

Perfusion. Guinea-pigs were anesthetized by intraperitoneal injection of 45 mg Mebumal[®]/kg body wt (ACO, Sweden). Perfusion with 0.1 M sodium citrate, pH 7.4, and 0.15 M NaCl, at a flow rate of 0.5–0.7 l/hr was carried out as described by Åkerström (1983); inflow through the left cardiac ventricle, out-flow through v. cava inferior. After perfusion the liver immediately was removed, trimmed and frozen at -80°C until used.

Homogenizing and solubilization. All homogenizing and solubilization operations were performed at 4°C . The livers were thawed and cut into small pieces. Five grams liver was homogenized (five strokes, 2000 rpm) in 5 ml ice-cold 0.5 M Tris-HCl, pH 8.5, containing 0.25 M sucrose, 1 mM EDTA and 1 mM PMSF (Sigma Chemical Co., St. Louis, MO). Another 15 ml ice-cold buffer was added and the homogenate was centrifuged at 10,000 g for 20 min. The supernatant was further centrifuged at 105,000 g for 60 min. The new supernatant was discarded and the pellet, constituting the crude membrane fraction, was solubilized with 0.2% (w/v) C_{12}E_8 in the homogenizing buffer.

Immunoprecipitation

Immunoprecipitation of ^{125}I -labeled antigen with goat-anti-guinea-pig $\beta_2\text{m}$ in the first step and rabbit-anti-goat IgG in the second was performed as has been reported (Åkerström *et al.*, 1979). The immune complexes were isolated by centrifuging on a discontinuous sucrose gradient (Taylor and Schimke, 1973).

SDS-PAGE

SDS-PAGE was run in rods (Neville, 1971). Total acrylamide concn in the separation gels was 13.7% and the cross-linking 3.7%. The rods were sliced into 1-mm fractions. BSA (mol. wt 67,000) and urinary guinea-pig $\beta_2\text{m}$ (mol. wt 11,500) were used as markers.

RESULTS

Isolation of non-associated $\beta_2\text{m}$ from guinea-pig serum

To remove the bulk of plasma proteins and especially $\beta_2\text{m}$ in association with other molecules, serum from seven guinea-pigs diluted threefold with Tris-buffered saline (pH 8.0), was subjected to ultrafiltration with an $\frac{8}{32}$ in. dialysis tubing. This tubing filters unbound $\beta_2\text{m}$ while larger molecules and complexes are retained.

A concn range of 3.4–6.6 mg/ml was estimated from analysis of $\beta_2\text{m}$ in nine guinea-pig sera when a fixed dilution, close to 50% inhibition, was chosen. Approximately 10–15% of the total serum $\beta_2\text{m}$ was recovered in the ultrafiltrate.

Gel filtration. In order to remove remaining high mol. wt complexes of $\beta_2\text{m}$ the filtered low mol. wt fraction was concentrated and subjected to gel filtration on Sephadex G-200 superfine. $\beta_2\text{m}$ was eluted in two regions (Fig. 1). The first peak, detectable by a radioimmunoassay, corresponds to that of $K_{av} = 0.33$ found in untreated serum (Löfdberg *et al.*, 1981). The second represents uncomplexed $\beta_2\text{m}$ and was analysed by a single radial immunodiffusion. The normal ratio between these two peaks in serum is usually 1:2; the monomeric $\beta_2\text{m}$ peak being the larger. After the ultrafiltration the ratio was of the order of 1:10³.

Ion exchange chromatography. The combined fractions from the gel filtration (Fig. 1) were concentrated, dialyzed against 0.02 M Tris-HCl, pH 8.0, and then applied to a column of Sephadex-DEAE A-50, equilibrated with the same buffer. $\beta_2\text{m}$ was eluted with a linear sodium chloride gradient. $\beta_2\text{m}$ appeared in two peaks at NaCl concns of 25 and 40 mM (Fig. 2B). Approximately the same amount of $\beta_2\text{m}$ was detected in the two peaks. For comparison urine from a normal guinea-pig was subjected to gel filtration and ion exchange chromatography in a similar way as serum (Fig. 2A).

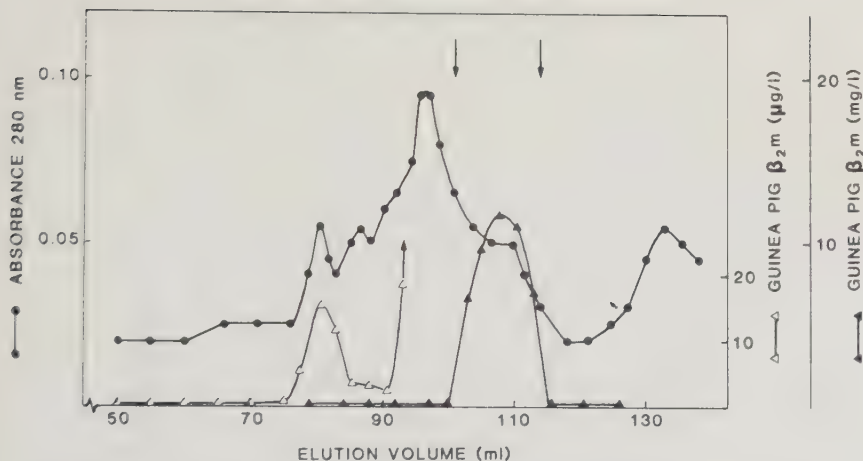


Fig. 1. Gel chromatography of the low mol. wt fraction from the ultrafiltration. The filtrated fraction of 93 ml guinea-pig serum was concentrated to 1.0 ml and applied to a column of Sephadex G-200 superfine (94×1.4 cm), equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN_3 . Fractions of 1.3 ml were collected at a flow rate of 1.7 ml/hr. The distribution of protein (●—●), guinea-pig β_2 m determined by a radioimmunoassay (Δ — Δ), and guinea-pig β_2 m measured by single radial immunodiffusion ($\blacktriangle$ — $\blacktriangle$) are shown. The arrows indicate how the fractions were combined.

Isolation of guinea-pig β_2 m from solubilized liver membranes

Crude membrane fractions from perfused livers were prepared as outlined in Materials and Methods. The efficiency of the detergents $\text{C}_{12}\text{E}_{12}$, Nonidet P40 and deoxycholate to solubilize and release "free" β_2 m from membranes was compared. Similar results for all three were obtained by determining the β_2 m content in solubilized membranes and by estimating the amount of non-associated β_2 m after gel filtration (data not shown). $\text{C}_{12}\text{E}_{12}$ was chosen as it is a non-ionic detergent and does not absorb light at 280 nm.

The solubilized membranes contained about 75 mg protein/g perfused liver tissue as measured by the Folin method. About 0.01% of the total protein was estimated to be β_2 m.

First gel filtration. Solubilized liver membranes representing 7.5–10 g liver tissue were diluted twice and subjected to gel chromatography on Sephadex G-200 (Fig. 3). Most of the protein eluted with the void vol while the main part of β_2 m emerged at an approximate K_{av} value of 0.76. Purified urinary β_2 m in serum elutes at the same position regardless the presence of detergent or not. In some experiments small amounts of β_2 m were recovered also in the void vol.

Second gel filtration. In order to remove most of the detergent and the sucrose, the combined and concentrated β_2 m fractions from five gel chromatographies were subjected to gel filtration on Sephadex G-100 (135×3.6 cm), equilibrated in a

detergent-free buffer (0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN_3). Almost all the β_2 m was eluted in the uncomplexed form. Only a small amount appeared in complexed or aggregated form at $K_{av} = 0.1$ (data not shown).

Ion exchange chromatography. Next the combined and conc. β_2 m fraction was dialyzed against 0.02 M Tris-HCl, pH 8.0. Ion exchange chromatography on Sephadex-DEAE A-50 was performed as described for serum β_2 m. In contrast to the serum experiment the β_2 m was eluted only as a single peak (Fig. 2C) at an NaCl concn of 20 mM. At the position of the second urinary peak, trace amounts of β_2 m could be noted in some experiments.

Immunoprecipitation and SDS-PAGE

Aliquots of the combined, conc. fractions of the respective gel filtrations of serum and the solubilized liver membranes were radiolabeled and then analysed on SDS-PAGE (Fig. 4A and D). Only 3.3 and 4.6% respectively was precipitated with an antiserum against urinary guinea pig β_2 m (Fig. 4B and E). The same antiserum precipitated about 70% of radiolabeled purified urinary β_2 m (data not shown). As could be expected no size differences were seen between the β_2 ms from different sources. Also, the experiment revealed no size-heterogeneity of β_2 m in serum (Fig. 4B).

DISCUSSION

When guinea-pig β_2 m was isolated and characterized from urine of outbred animals two forms of the

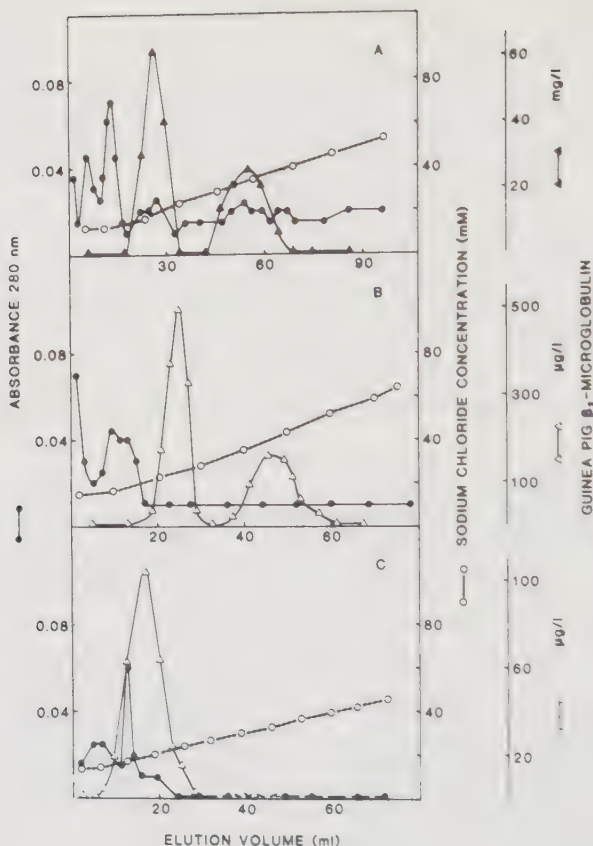


Fig. 2. Ion exchange chromatography of the combined and conc. fractions of gel filtrations of normal guinea-pig urine (A), guinea-pig serum (B), and solubilized guinea-pig liver membranes (C). The samples were dialyzed against 0.02 *M* Tris-HCl, pH 8.0, and applied to columns of 6×5 cm of Sephadex DE AE A-50, equilibrated with the same buffer. The proteins (●—●) were eluted with a linear gradient of sodium chloride, 0–0.1 *M* (○—○). Guinea-pig β_2 m was determined by single radial immunodiffusion (▲—▲) or a radioimmunoassay (△—△).

protein were isolated (Cigén *et al.*, 1978). They differed in electrophoretic mobility and this was ascribed to the finding that the major (basic) form apparently had one lysyl residue more than the minor (acidic) one. It was discussed that the two forms could reflect genetic polymorphism, but the minor component could also be a result of post-synthetic modification. Whatever the case is, it is of interest to investigate the distribution of the two forms, to see if they are detectable in other biological fluids or tissues than urine.

In this report the charge of partially isolated, unassociated β_2 m in guinea pig serum and on liver cell membranes have been analysed and compared with that of urinary β_2 m. Only one form was found

on liver cell membranes, while two differently charged forms were detected in serum

To separate the uncomplexed serum β_2 m from most of the other serum proteins, including β_2 m in association with high mol. wt molecules (Natori *et al.*, 1976; Kvist and Peterson, 1978; Lögdberg *et al.*, 1979; Björck and Berggård, 1981), ultrafiltration with a mol. wt cut-off at 30,000–40,000 was used. The technique is rapid compared to gel filtration, as much larger vols can be filtered at the same time. The efficiency of the method was difficult to estimate. Using radioimmunoassay, the concn of β_2 m in serum showed a marked dependence on the degree of dilution, probably reflecting the high proportion of β_2 m (20–40%) associated with other substances (Lögdberg

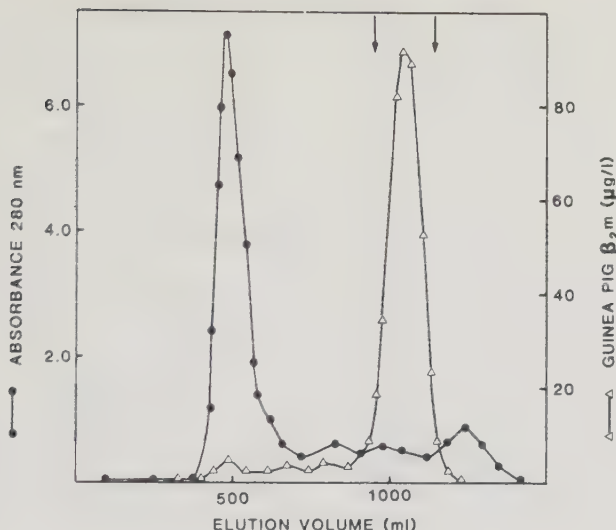


Fig. 3. Gel filtration of solubilized guinea-pig liver membranes. Ten millilitres (equivalent to 10 g perfused liver tissue) of the solubilized membrane fraction was diluted twice with 1 mM EDTA, 1 mM PMSF and 0.2% $C_{12}E_{12}$ in distilled water to reduce the sucrose and Tris-HCl concns. The membranes were separated on a column (140 $\times$ 3.5 cm) of Sephadex G-200, equilibrated with 0.02 M Tris-HCl, pH 8.5, containing 0.1 M sucrose, 1 mM EDTA, 1 mM PMSF and 0.2% $C_{12}E_{12}$. Eleven-millilitre fractions at a flow rate of 15 ml/hr were collected. Protein was measured by reading the absorbance at 280 nm (●—●) and guinea-pig β_2 m ($\triangle$ — $\triangle$) was determined by a radioimmunoassay. The fractions were combined as the arrows indicate.

et al., 1981; unpublished observations). However 10–15% roughly estimates the recovered β_2 m.

Gel filtration was performed to remove possible remaining high mol. wt β_2 m. Thereafter, serum β_2 m was assayed for charge heterogeneity by ion exchange chromatography. It eluted in two peaks at approximately the same sodium chloride concn as the urinary counterparts. This suggests that the serum β_2 m forms are similar to those detected in urine.

To analyse the liver membrane associated β_2 m, crude membrane fractions, solubilized in $C_{12}E_{12}$, were prepared. After partial isolation of the β_2 m on gel filtration, the membrane β_2 m was analysed on ion exchange chromatography. In contrast to serum β_2 m, membrane β_2 m eluted only in one peak corresponding to the first serum peak. The traces, sometimes found at the base-line at higher sodium chloride concns could be a remnant of serum due to imperfect liver perfusion. If it is of hepatic origin it would represent a far smaller fraction of the total β_2 m than is the case in serum or urine.

Recently genetic variants of β_2 m have been described in certain mouse strains (Goding and Walker, 1980; Michaelson *et al.*, 1980; Gates *et al.*, 1981). Net charge heterogeneities in β_2 m have also been reported in urine from man (Hall *et al.*, 1977; Tanahashi *et al.*, 1979b), the rat (Lögdborg *et al.*, 1979) and the rabbit

(Björck and Berggård, 1981), and in human colostrum (Tanahashi *et al.*, 1979a).

The fact that I only find one β_2 m form on liver cell membranes but two in serum is probably not a result of genetic polymorphism of the kind described in the murine system. In the mouse both forms are expressed on the cell surface in heterozygous animals, but only one of the forms in homozygous ones. This would mean that if the two guinea-pig β_2 m forms are products of different β_2 m alleles the pooled liver material has been sampled exclusively from animals homozygous for one of the alleles. This seems improbable as two forms of guinea-pig β_2 m have always been found in urine, collectively or individually collected (Cigén *et al.*, 1978; unpublished observations), as well as in pooled serum. This could also be studied by analysing β_2 m on cell membranes and urine from inbred guinea-pig strains. Such studies are under way.

The origin of the two forms is more likely of post-translational nature. It is reasonable to believe that the acidic β_2 m form lacks the C-terminal residue reported by Wolfe and Cebra (1980) to be lysine. However, it seems unlikely that the lysyl residue is lost in serum through proteolysis as strong denaturing agents, like SDS, are needed *in vitro* to release the C-terminal lysin by carboxypeptidase B (Wolfe and Cebra, 1980). If this is true also *in vivo*,

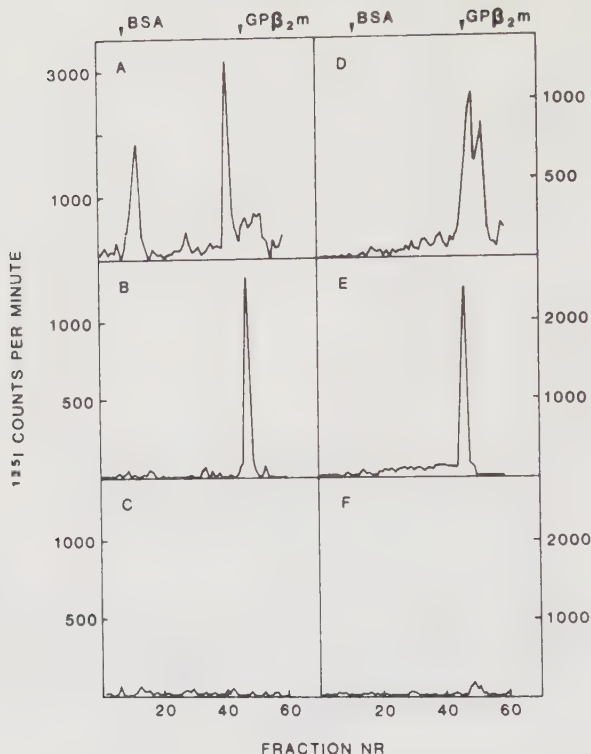


Fig. 4. SDS-PAGE of the ^{125}I -labeled combined fractions of the gel filtrations of serum (A-C) and solubilized liver membranes (first gel filtration) (D-F). (A and D) not precipitated; (B and E) precipitated with goat-anti-guinea-pig $\beta_2\text{m}$; (C and F) precipitated with normal goat serum. The mobilities of BSA and urinary guinea-pig $\beta_2\text{m}$ are indicated by the arrows.

$\beta_2\text{m}$ must regain its tertiary structure after digestion as it reacts well with antibodies made against the basic $\beta_2\text{m}$ in assays like single radial immunodiffusion and radioimmunoassay (Figs 1-3). Thus it seems plausible that the modification occurs before the folding of the protein.

It is also unknown whether the guinea-pig liver is unique in producing only the basic $\beta_2\text{m}$ form, which means the acidic $\beta_2\text{m}$ has to be produced by other tissues. Another possibility is that both forms are made in the liver but only the basic form associates to other membrane structures while the acidic $\beta_2\text{m}$ form is secreted.

Acknowledgements—This work was supported by grants from the Swedish Medical Research Council (Project No. 512) and from the Medical Faculty, University of Lund, Sweden. I wish to thank Ms Elisabeth Persson for excellent technical assistance.

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Binding of β_2 -Microglobulin by Heterologous Sera

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Lögdberg, L., Cigén, R. & Björck, L. Binding of β_2 -Microglobulin by Heterologous Sera. *Scand. J. Immunol.* 13, 237-243, 1981.

Purified human, rat or guinea-pig β_2 -microglobulin (β_2m) was mixed with sera from guinea-pig, rat, mouse, rabbit, horse, goat, cow, rhesus monkey or man. The mixtures were incubated at 37°C for various lengths of time. When the sera were separated by gel-chromatography on Sephadex G-200, β_2m was traced not only in 'free' form but also in fractions with higher molecular weights. Evidence is presented suggesting that heterologous β_2m binds to β_2m -containing molecules in sera by exchange with the homologous counterpart.

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Interest has focused on the low molecular weight protein β_2 -microglobulin (β_2m) [3], mainly because [for references see 2, 20]: (1) β_2m is structurally related to immunoglobulins and has been called a 'free immunoglobulin domain'; (2) β_2m is a constant part of the serologically defined strong transplantation antigens in man (HLA-A, -B and -C antigens), mouse (H-2 K, D and L antigens), and several other species; (3) β_2m is also claimed to be associated with several other molecules on cell surfaces and in biological fluids; (4) studies with antibodies directed against β_2m have indicated a role for β_2m in the mechanism of lymphocyte activation; (5) the β_2m concentration in biological fluids changes in various pathological conditions, notably diseases of the kidney, inflammatory diseases, and various malignancies.

One group showed that human β_2m binds to lymphocytes from mouse, rat and guinea-pig [1] and that the receptor for human β_2m on mouse lymphocytes consists, at least partly, of H-2 antigen molecules [10]. This report demonstrates a similar phenomenon, namely that β_2m binds to components in heterologous sera.

MATERIALS AND METHODS

β_2m and antisera against β_2m . Human β_2m [3], guinea-pig β_2m [5] and rat β_2m [16] were purified in this

laboratory. We used one rabbit anti-human β_2m [3], one goat anti-human β_2m (prepared as in Ref. 4), one goat anti-guinea-pig β_2m [4], one goat anti-rat β_2m [16] and one rabbit anti-rat β_2m [16].

Sera. We used sera from nine mammalian species belonging to five orders: man, rhesus monkey (Primates), guinea-pig, rat, mouse (*Rodentia*), rabbit (*Lagomorpha*), horse (*Perissodactyla*), goat and cow (*Artiodactyla*).

Radioimmunoassay (RIA) of β_2m . β_2m 's were ^{125}I -radio-labelled by the chloramine-T method [9] and separated from free ^{125}I by gel filtration on Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden). RIA for β_2m was performed as described by Plesner *et al.* [22], using polyethyleneglycol exclusion to separate immune complexes from unbound β_2m . Homologous β_2m RIAs were based on the reactions between ^{125}I -labelled human, rat or guinea-pig β_2m 's and their specific goat antisera, respectively; heterologous RIA [8] was based on the reaction between rabbit anti-rat β_2m and ^{125}I -labelled human β_2m (heterologous RIA-1) or the reaction between rabbit anti-human β_2m and ^{125}I -labelled guinea-pig β_2m (heterologous RIA-2). The two heterologous RIAs were selected as being highly sensitive for demonstration of cross-reactions. The detection limit of the homologous RIAs varied between batches of labelled protein (0.2-5 $\mu g/l$).

Incubation of β_2m with heterologous serum. Purified β_2m was mixed with heterologous serum, usually 1-3 $\mu g/ml$ serum, and incubated at 37°C for 1-2 days, unless otherwise stated. The incubations were interrupted by the application of the mixtures to columns of Sephadex G-200.

Separation of serum by gel chromatography. Columns of Sephadex G-200 (Pharmacia), with lengths of 90-130 cm and volumes of 60-180 ml, were equilibrated with 0.02 M Tris-HCl buffer + 0.15 M NaCl + 0.02%

NaN_3 , pH 8.0, and used for serum separation. In a few experiments a larger Sephadex G-200 column (5×135 cm) was used, equilibrated with the same buffer. The protein distribution was determined by measuring the absorbance at 280 nm. $\beta_2\text{m}$ was traced by homologous or heterologous RIA.

RESULTS

Rat $\beta_2\text{m}$ mixed with horse serum and incubated at 37°C for 24 h and then chromatographed on Sephadex G-200, was not solely recovered in 'free' form but also in two high molecular weight peaks, B and C, with average K_{av} values of 0.09 and 0.42, respectively (Fig. 1). The peak of 'free' $\beta_2\text{m}$ is designated D.

Influence of incubation temperature, incubation time, and $\beta_2\text{m}$ concentration on the complex formation of rat $\beta_2\text{m}$ in horse serum

Incubation temperature (24 h; $2\mu\text{g}$ rat $\beta_2\text{m}/\text{ml}$). With incubation at 4°C , or less, all rat $\beta_2\text{m}$ was eluted in 'free' form. Rat $\beta_2\text{m}$ could also be detected as a peak B after incubation at 20°C .

However, after incubation at 37°C , the amounts of rat $\beta_2\text{m}$ present in peak B were markedly increased; in addition, rat $\beta_2\text{m}$ was also detected as a peak C.

Incubation time (37°C ; $2\mu\text{g}$ rat $\beta_2\text{m}/\text{ml}$). If horse serum was separated immediately after mixture with rat $\beta_2\text{m}$, the rat $\beta_2\text{m}$ was only recovered as a peak D. Already after 3 h of incubation, significant amounts of complexed rat $\beta_2\text{m}$ could be detected. Incubation times of more than 2 days increased the amounts of complexed rat $\beta_2\text{m}$ only to a small extent. However, prolonged incubation time led to decrease of peak B and increase of peak C.

Rat $\beta_2\text{m}$ concentration (37°C ; 24 h). At $0.4\mu\text{g}$ rat $\beta_2\text{m}/\text{ml}$, complexed rat $\beta_2\text{m}$ was barely detectable (in these experiments, the detection limit of the RIA lay between 2 and $5\mu\text{g}/\text{l}$). At $2\mu\text{g}/\text{ml}$, about one fourth of the rat $\beta_2\text{m}$ could be detected in well-defined peaks B and C. At $10\mu\text{g}/\text{ml}$, the absolute amounts of complexed rat $\beta_2\text{m}$ further increased, but its percentage decreased.

On the basis of these experiments, we used incubations at 37°C for 1–2 days with 1–3 μg $\beta_2\text{m}/\text{ml}$ in most of the following experiments.

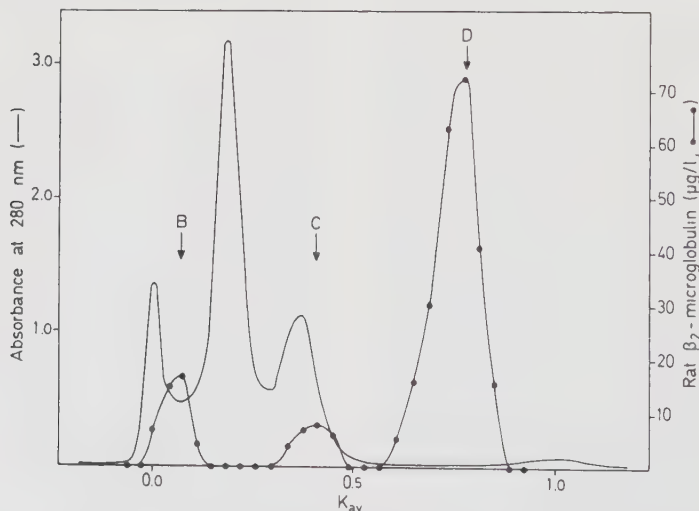


FIG. 1. Horse serum was incubated with rat $\beta_2\text{m}$ ($2\mu\text{g}/\text{ml}$) at 37°C for 24 h. $450\mu\text{l}$ of the mixture was applied to a column (1.1×93 cm) of Sephadex G-200, equilibrated with 0.02 M Tris-HCl, pH 8.0, containing 0.15 M NaCl and 0.02% NaN_3 . The flow rate was 2 ml/h and 1-ml fractions were collected. The protein distribution was measured as absorbance at 280 nm. Rat $\beta_2\text{m}$ was traced by homologous rat $\beta_2\text{m}$ RIA. For convenience, the elution volumes were transformed to K_{av} values. The $\beta_2\text{m}$ peaks are designated with capital letters (B, C, D) as defined in the text.

Interaction of β_2m with various heterologous mammalian sera

We screened the interaction of human, rat or guinea-pig β_2m with several mammalian sera. Peaks of complexed material were found in three regions after Sephadex G-200 gel chromatography: (A) at V_0 (peak $K_{av} = 0$); (B) between the first and second protein peaks (peak $K_{av} = 0.08-0.15$); and (C) around the third protein peak (peak $K_{av} = 0.32-0.42$). In addition, we frequently detected β_2m peaks between peaks C and D. These were not as readily reproducible as the others. They were thought to represent

self-association of β_2m and were not further investigated.

The peaks were scored as - (not detected); (+) (β_2m detected but peak not distinct, because of low amounts, close to the detection limit, or poor resolution from background level); + (distinct, well-defined β_2m -peak containing < 10% of total β_2m); or ++ (β_2m peak containing > 10% of total β_2m). Table I gives a compilation of the results. It also gives the elution profile of homologous β_2m in mouse, horse and cow sera, as detected by heterologous RIA. Of the various sera, only mouse serum could inhibit the homologous rat β_2m RIA. The inhibitions

TABLE I. Binding of β_2m by heterologous sera

Serum	β_2m addition	Peak A	Peak B	Peak C
Man	Rat β_2m	+	(+)	(+)
	Guinea-pig β_2m	(+)	(+)	++
Rhesus monkey	Human β_2m	(+)	(+)	(+)
	Rat β_2m	(+)	(+)	+
	Guinea-pig β_2m	(+)	(+)	+
Rat	Human β_2m	(+)	(+)	(+)
	Guinea-pig β_2m	(+)	-	+
Guinea-pig	Human β_2m	(+)	-	+
	Rat β_2m	+	-	++
Mouse	Human β_2m	-	++	++
	Rat β_2m	-	++	++
	Guinea-pig β_2m	-	++	++
Heterologous RIA-1		-	++	++
Rabbit	Human β_2m	(+)	+	(+)
	Rat β_2m	(+)	(+)	+
	Guinea-pig β_2m	+	(+)	++
Cow	Human β_2m	(+)	(+)	(+)
	Rat β_2m	-	(+)	+
	Guinea-pig β_2m	-	-	(+)
Heterologous RIA-1		-	-	(+)
Heterologous RIA-2		-	-	-
Horse	Human β_2m	-	++	-
	Rat β_2m	-	++	+
	Guinea-pig β_2m	-	++	++
Heterologous RIA-1		-	++	++
Goat	Human β_2m	(+)	(+)	++
	Rat β_2m	+	(+)	++
	Guinea-pig β_2m	+	+	++

Human, rat or guinea-pig β_2m was added to the various sera to final concentrations of 1-3 $\mu g/ml$. The mixtures were incubated at 37°C for 1-2 days and then chromatographed on columns of Sephadex G-200. The β_2m content of the fractions was determined by RIAs homologous to the species of β_2m added. β_2m -containing peaks were integrated to measure their total β_2m content. The presence of high molecular weight β_2m peaks in regions A, B, and C are indicated by a score: - = not detected; (+) = β_2m detected but peak not distinct; + = distinct β_2m peak containing < 10% of total β_2m ; ++ = β_2m -peak containing > 10% of total β_2m . In a few experiments, sera were separated on Sephadex G-200 after no addition of heterologous β_2m . The β_2m distribution was then determined by heterologous RIA.

caused by such cross-reactive materials, however, were low compared with that caused by complexed rat β_2 m. Incubations with mouse or horse sera gave similar elution profiles, with prominent peak B, more (mouse) or less (horse) prominent peak C, and no peak A (cf. Fig. 1). In the other sera, complexed β_2 m was preferentially detected in region C with various degrees of minor amounts in the other two regions (cf. Fig. 2). The elution profiles for the various β_2 m's were very similar within each serum. It should be noted that the location of both peak B and peak C varied between, but was reproducible within, species.

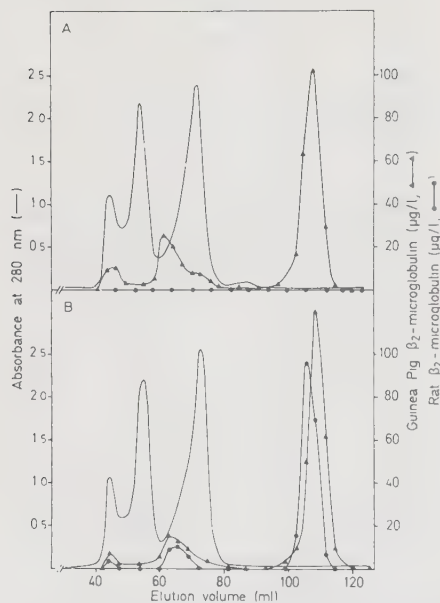


FIG. 2. Guinea-pig serum (A). Guinea-pig serum incubated at 37°C for 24 h with rat β_2 m (1 μ g/ml) (B). 750 μ l of each was separated on a column (1.4 $\times$ 90 cm) of Sephadex G-200 superfine, equilibrated with 0.02 M Tris-HCl + 0.15 M NaCl + 0.02% NaN₃, pH 8.0. The flow rate was 2 ml/h, and 1.5-ml fractions were collected. The protein distribution was measured as absorbance at 280 nm. The rat and guinea-pig β_2 m in the eluates were traced by the respective homologous β_2 m RIAs.

Re-chromatography of selected peaks

Fractions comprising peak B or C of rat β_2 m-containing horse serum were pooled, concentrated, and re-chromatographed on Sephadex

G-200. All of the peak C material eluted again in region C. More interesting, only a part of the peak B material emerged in region B, the rest eluting at region C.

When peak C of rat β_2 m-incubated rabbit serum was similarly re-chromatographed in large amounts, the bulk of the material emerged in region C. However, a small peak A also appeared.

Effect of guinea-pig β_2 m on the complex formation of rat β_2 m in guinea-pig serum

Guinea-pig serum (Fig. 2A) and guinea-pig serum incubated with rat β_2 m (Fig. 2B) were separated by Sephadex G-200 gel chromatography. The rat and guinea-pig β_2 m content of the fractions were determined by the respective homologous RIAs. It is notable that the elution of complexed rat β_2 m coincides with the elution of high molecular weight guinea-pig β_2 m complexes (Fig. 2B). When we added large amounts of purified guinea-pig β_2 m (1 mg/ml) to the incubation mixture of rat β_2 m and guinea-pig serum, the rat β_2 m of the resulting G-200-separation was almost exclusively confined to peak D (not shown).

DISCUSSION

The results presented in this paper indicate that purified β_2 m binds to components in heterologous sera. Bound β_2 m is detected as peaks in three regions of the various sera (regions A–C as defined above).

The binding profiles show striking similarities to the distribution profile of homologous β_2 m in the various sera, as these have been reported for mouse [13, 18], man [17], rat [16], rabbit [7, Björck, L. & Berggård, I., unpublished] and guinea-pig [present paper]. Moreover, in horse serum, evaluated by heterologous RIA, the β_2 m distributed in the same pattern as the heterologous β_2 m-binding profile (Table 1). When ¹²⁵I-labelled rat β_2 m was mixed with rat serum incubated at 37°C for 24 h and then separated on Sephadex G-200, we earlier found that the radioactivity would be recovered in three peaks, corresponding to the rat β_2 m detectable in rat serum with RIA [16]. These results were interpreted as due to an exchange of the rat β_2 m in the high molecular weight β_2 m-containing materials with ¹²⁵I-labelled rat

β_2m [16]. Thus, it seems probable that heterologous β_2m binds to high molecular weight β_2m -containing molecules through exchange with the homologous counterpart.

Further support for the exchange hypothesis was obtained in experiments in which rat β_2m was bound by guinea-pig serum (Fig. 2). Addition of large amounts of purified guinea-pig β_2m almost completely blocked the binding of rat β_2m to guinea-pig serum. In addition, Fig. 2B shows that similar amounts of rat and guinea-pig β_2m leads to quantitatively comparable distribution profiles of the two proteins in guinea-pig serum. This suggests that the β_2m -binding materials in peaks A and C have affinities of the same magnitude for rat and guinea-pig β_2m , respectively. Thus, our data indicate that there is an evolutionary conservatism in the binding sites of β_2m and the complementary sites of the β_2m -binding molecules, and that the heterologous binding represents a form of cross-reactivity.

Discussing the mechanisms for the binding of human β_2m to histocompatibility antigens on mouse and human lymphocytes, Hester *et al.* [10] suggested two possibilities: (1) more than one β_2m molecule might bind to a single histocompatibility antigen heavy chain, and (2) there exist on the cell surface histocompatibility antigens lacking β_2m and therefore capable of binding β_2m . The two models do not seem to be probable candidates to explain the binding of β_2m by heterologous sera. Serum contains considerable amounts of unbound β_2m , and such mechanisms would accordingly be expected to be saturated. Thus, an exchange model based on dynamic equilibrium reactions appears to be a more probable explanation of this phenomenon. Exchange could also be the mechanism behind the cytophilic properties of human β_2m for heterologous or activated homologous lymphocytes.

The rat β_2m -containing peak B of horse serum was unstable and gave a peak in region C when re-chromatographed. This lack of stability, also supported by the temporal binding pattern, has previously been noted for the homologous β_2m peak B of mouse serum [13]. In that report, the resulting peak C material had a structure closely resembling H-2 antigens but lacked alloantigenic activity. A small part of the rat β_2m -containing peak C of rabbit serum eluted as peak A material when re-chromato-

graphed. One may speculate that a flow—peak B $\rightarrow$ peak C $\rightarrow$ peak A—exists, and a heterologous binding of β_2m to peak B could then explain our results. With homologous RIA, significant amounts of peak B β_2m have been found in the mouse [13, 18] and man [17], but not in the rabbit [7; Björck, L. & Berggård, I., unpublished], or guinea-pig [present paper]. We could not detect peak B β_2m in rat serum [16]; recently, another group found a peak B β_2m in sera of Hooded Lister rats [12]. The reason for this is unknown, but possible explanations are that peaks B of the latter species are rapidly degraded to peak C materials, or that antigenic β_2m determinants of these materials are hidden.

A second type of molecules that could be responsible for heterologous β_2m -binding are the major histocompatibility antigens (HLA-A, -B, and -C antigens and their animal homologues) [10]. This is also suggested by studies on man/mouse cell hybrids [6, 11]. Mouse β_2m apparently could replace human β_2m in the HLA antigens expressed on the cell surface of the hybrid cells. Our own recent demonstration that purified papain-solubilized AgB antigens can bind heterologous β_2m further supports this notion [15]. These materials would be expected to elute in region C. Their different tendency to aggregate and form dimers [26] could explain the different location of the peak C, responsible for heterologous β_2m -binding, of the various species. Peak C of guinea-pig serum would then mainly consist of the dimer, with only small amounts of monomer (Fig. 2A), whereas peak C of horse serum represents the monomer (Fig. 1). However, so far we have not been able to precipitate, with relevant alloantisera, ^{125}I -labelled guinea-pig or human β_2m bound to peak C of serum from inbred Wistar-Furth rats (unpublished data; cf. Ref. 15). This very preliminary observation suggests that the main part of the β_2m -binding molecules in heterologous sera are devoid of alloantigenic activity. Such molecules could possibly also explain part of the cytophilic properties of β_2m [23].

β_2m , immunoglobulins, and major histocompatibility antigens appear to share a common evolutionary origin [19, 21, 24, 25]. Thus, it is of interest to trace, isolate, and characterize β_2m in lower species. The heterologous RIA described by Gordon & Kindt [8] should be a valuable tool in such studies. The heterologous binding of β_2m , as described in the present

paper, could be a helpful supplement to the heterologous RIA. This is exemplified by cow serum (Table I), where two sensitive heterologous RIAs failed to demonstrate unambiguously anything but 'free' β_2 m. A clear peak C material, however, was demonstrated by the heterologous binding technique.

β_2 m is apparently important for the conformation of major histocompatibility antigens [14, 26]; the loss of β_2 m is often followed by diminished alloantigenic activity. It would be interesting to investigate whether heterologous β_2 m exchange in β_2 m-containing molecules leads to altered conformation, detectable with biochemical or immunological methods.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (Project No. 512) and the Medical Faculty, University of Lund.

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Received 13 June 1980

Received in revised form 29 September 1980

DIFFERENCE IN BINDING CAPACITY BETWEEN STRUCTURALLY DIFFERENT
GUINEA-PIG β_2 -MICROGLOBULIN FORMS AND HETEROLOGOUS SERA

Running title: Guinea-pig β_2 -microglobulin forms and heterologous sera

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SUMMARY

The ability of two forms (basic and acidic) of guinea-pig β_2 -microglobulin (β_2 m) with different C-terminal amino acids to interact with heterologous sera was tested and compared.

β_2 M of either form was incubated with horse serum for various periods of time whereupon the incubations were subjected to gelfiltration and the eluted β_2 m was determined by radioimmunoassay.

β_2 M in complexes with serum molecules was found in two size regions (I and II). In all incubations more basic than acidic β_2 m was bound. The distribution between the regions differed in that more basic than acidic β_2 m always was detected in region I but the reverse was found in region II after 3-4 hours of incubation. The results also demonstrate that region I complexes decompose to region II complexes.

Similar differences in binding capacity between the two β_2 m components were also obtained from incubations with mouse serum.

The discrepancy in binding to heterologous sera in relation to the C-terminal structural difference between the two guinea-pig β_2 m forms is discussed.

INTRODUCTION

β_2 M is a polypeptide with mol. wt. of 11 800. It was first isolated by Berggård and Bearn (1968) from urine from patients with tubular dysfunctions. β_2 M occurs in all biological fluids and on nucleated cells and it is known for its non-covalent association to MHC Class I antigens (cf Berggård *et al.*, 1980). Its structural homology to domains of immunoglobulins and MHC Class I and II antigens (Smithies and Poulik, 1972; Orr *et al.*, 1979; Trägårdh *et al.*, 1979; Larhammar *et al.*, 1981) point to a common evolutionary origin for these molecules.

Much of later years interest in β_2 m has concentrated on its interaction with MHC related molecules. Homologous as well as heterologous β_2 m is able to exchange with preexisting homologous β_2 m in purified MHC Class I antigens (Hyafil and Strominger 1979; Lögdberg *et al.*, 1980; Schmidt *et al.*, 1981; Church *et al.*, 1982; Parham 1983). Such a mechanism is also

believed to explain the binding of β_2m to cell surfaces (Hester *et al.*, 1979; Lögdberg *et al.*, 1981 a; Bernabeu *et al.*, 1984; Kefford *et al.*, 1984) and in serum (Lögdberg *et al.*, 1981 b; Vincent *et al.*, 1981; Ramanathan *et al.*, 1982).

So far not much is known about the structures in β_2m that are responsible for the described interactions, although two tyrosine residues seem to be involved (Parker and Strominger 1983).

When guinea-pig β_2m was purified from urine, two forms of the protein were found that differed in amino acid composition (Cigén *et al.*, 1978). In sequence studies the amino acid difference was localized to the C-terminal amino acid where the basic guinea-pig β_2m form has lysine and the acidic form lacks this residue (Wolfe and Cebra 1980; Cigén to be published).

In this study I have quantitatively compared the ability of the two guinea-pig β_2m forms to interact with heterologous sera in order to see whether the C-terminal amino acid may be of any importance for such interactions. An implication of this has recently been given in that the acidic β_2m form not is found on membranes from liver tissue (Cigén 1983).

MATERIALS AND METHODS

Proteins, antisera and sera

Guinea-pig β_2m , both forms, were purified as has been described (Cigén *et al.*, 1978). Basic β_2m refers to the β_2m with a C-terminal lysyl-residue and acidic β_2m to the form lacking that residue (Cigén, 1984 in preparation). Polyclonal goat antiserum against the basic form of guinea-pig β_2m was prepared and tested as has been reported (Björck *et al.*, 1977). Horse serum was from Flow Laboratories, Ayrshire, Scotland. Mouse serum was obtained by orbital bleeding. Lyophilized mouse serum was purchased from Miles Laboratories Inc. Bovine serum for the radioimmunoassay was provided by a local slaughter house.

Radiolabelling of β_2m

The Chloramin-T method of Greenwood *et al.*, (1963) was used to label β_2m with ^{125}I (Amersham, U.K.). Free iodine was separated from the protein bound by dialysis against 0.02 M Tris-HCl, pH 8.0 with 0.15 M NaCl and 0.02% NaN_3 . For the radioimmunoassay 5 μg β_2m was labelled with 0.5 mCi ^{125}I to a specific activity of 10-25 mCi/mg β_2m . In the Scatchard analysis 0.6 mCi ^{125}I was used to label 10 μg of either β_2m form (specific activity: 6-10 mCi/mg basic β_2m ; 5-7 mCi/mg acidic β_2m).

Determination of β_2m and total protein concentrations.

Ultrafiltration of protein solutions

Radioimmunoassay was used to determine the concentrations of either form of guinea-pig β_2m ; the basic form was used as the radiolabelled antigen. The antiserum was that described above. A 0.1 M phosphate buffer, pH 7.4, containing 0.02% NaN_3 and 0.1% bovine serum albumin was used for the dilutions. The immunocomplexes were precipitated with bovine serum and polyethylene glycol (6000, KEBO, Sweden) (Plesner *et al.*, 1975). Detection limit was set to 10% of inhibition, which is equivalent to 0.5 $\mu g/ml$. Total protein concentrations were determined by the absorbance at 280 nm. Protein solutions were concentrated by ultrafiltration through Visking dialysis tubing 24/32 in. (Union Carbide Corp., Chicago IL) (Berggård 1961).

Incubation of heterologous sera with guinea-pig β_2m

Aliquots of 0.5 ml of horse or mouse serum were incubated with 1.0 μg of either β_2m form at 37°C. Incubation periods are indicated in Results. The incubations were followed by gel chromatography on a column (1.4 x 105 cm) of Sephadex G-200 equilibrated with 0.02 M Tris-HCl, pH 8.0, with 0.15 M NaCl and 0.02% NaN_3 . Flow rate was 1.6 ml/h and the gelfiltrations were performed at 4°C. The fractions were then analysed for total protein and β_2m . The amount of β_2m in each region (defined in Fig. 2) was calculated by triangulation.

Determination of affinity constant and number of binding sites

Region I, first part, (see Fig. 2) of chromatographed horse serum, equivalent to 0.5 ml, was mixed with varying amounts of ^{125}I - β_2m . Volumes were corrected to 0.5 ml with the phosphate buffer used in the

radioimmunoassay. Samples with no horse serum were also prepared. The incubations were left for 3 h in 37°C. $\beta_2\text{m}$ -complexes were precipitated by bovine serum and polyethylene glycol (10%). After correction for background binding and the inaccessible fraction of ^{125}I - $\beta_2\text{m}$ (see Results) the radioactive count of bound and free $\beta_2\text{m}$ was used for Scatchard-analysis (Scatchard 1949).

RESULTS

Immunological similarity between the acidic and basic guinea-pig $\beta_2\text{m}$ forms

It is essential that the two forms of guinea-pig $\beta_2\text{m}$ react in a similar way in the radioimmunoassay to validate a quantitative comparison of their binding to heterologous sera.

Parallel plots were obtained when either form of radiolabelled guinea-pig $\beta_2\text{m}$ was titrated with decreasing amounts of goat-anti-guinea-pig $\beta_2\text{m}$, and the maximum binding of each form was set to 100% (not shown). However, a discrepancy was noted in the maximum binding of total ^{125}I - $\beta_2\text{m}$. The antibodies bound about 90% of the total basic and about 80% of the acidic ^{125}I - $\beta_2\text{m}$. Comparison of standard dilutions of purified $\beta_2\text{m}$ forms in a radioimmunoassay system also exhibited a high degree of parallelity

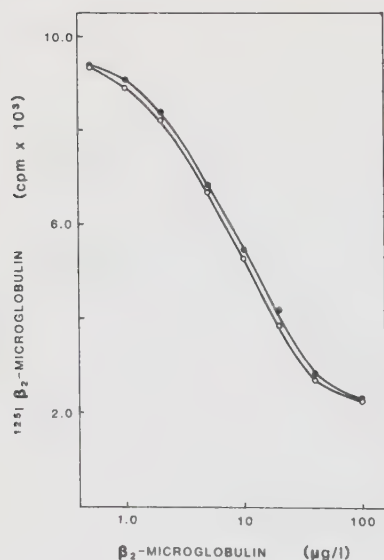


Fig. 1. Radioimmunoassay standard dilutions of purified basic (●—●) and acidic (○—○) guinea-pig $\beta_2\text{m}$. Acidic $\beta_2\text{m}$ was used as the radiolabelled antigen. The goat antiserum (1:60 000) was prepared against the basic guinea-pig $\beta_2\text{m}$ form.

irrespective of which form was used as the radiolabelled antigen. Fig. 1 shows the result when the acidic $\beta_2\text{m}$ serves as the radiolabelled $\beta_2\text{m}$.

Binding of guinea-pig $\beta_2\text{m}$ to horse serum

Gel chromatographies on Sephadex G-200 of horse serum incubated for 12 h, 37°C with each $\beta_2\text{m}$ form are shown in Fig. 2. $\beta_2\text{m}$ is recovered mainly in three regions (I-III) with approximative K_{av} ranges of 0.04-0.25 (I), 0.36-0.45 (II) and 0.69-0.85 (III). The third region represents uncomplexed $\beta_2\text{m}$.

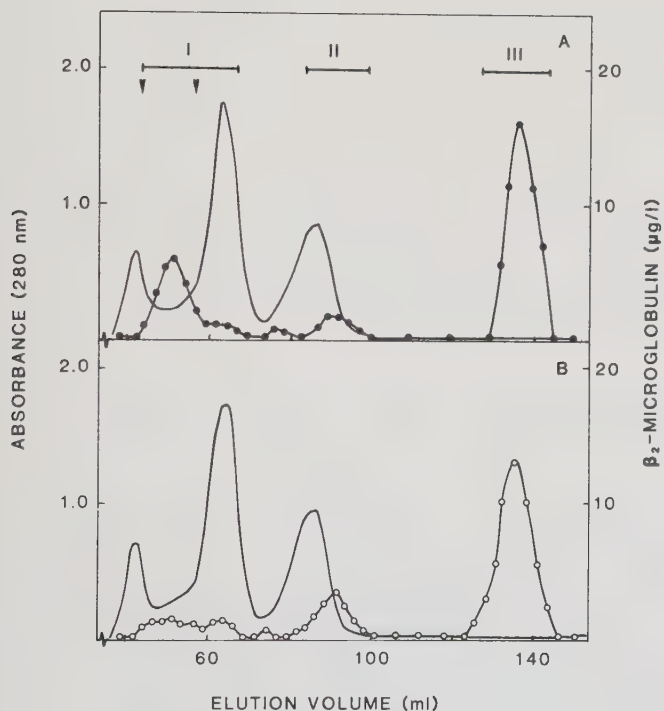


Fig. 2. Sephadex G-200 chromatography of 0.5 ml horse serum incubated for 12 h, 37°C , with 1.0 μg A) basic (●—●) or B) acidic (○—○) $\beta_2\text{m}$. Total protein was determined by the absorbance at 280 nm (—) and $\beta_2\text{m}$ by radioimmunoassay.

Differences in the interaction capacity are found both in region I and II. Region I seems to consist of two incompletely separated peaks, more prominent for the acidic $\beta_2\text{m}$, where the peaks are of similar sizes. In this region the difference in $\beta_2\text{m}$ -binding capacity is confined to the first peak.

Horse serum with no added guinea-pig $\beta_2\text{m}$ was gel filtrated and analysed with the guinea-pig $\beta_2\text{m}$ radioimmunoassay. No cross-reactivity with horse $\beta_2\text{m}$ was noted with the dilutions used.

The amounts of the two forms in region I and II after varying incubation periods are presented in Fig. 3. All incubations were made with the same batch of horse serum, and the total amount recovered $\beta_2\text{m}$ (including region III) was 19-22% in all gel filtrations. From Fig. 3A it is evident that region I binds almost twice as much basic as acidic $\beta_2\text{m}$. Both forms

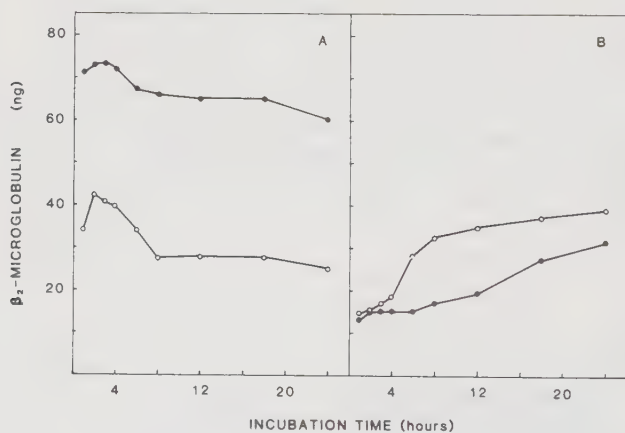


Fig. 3. Amount of $\beta_2\text{m}$ in region I (A) and II (B) (see Fig. 2 for definition) after gel filtrations on Sephadex G-200 of 0.5 ml horse serum incubated with 1.0 μg basic (●—●) or acidic (○—○) $\beta_2\text{m}$ for time periods indicated in the figure.

have a binding maximum after 2-3 hours. The amounts of bound $\beta_2\text{m}$ then decline to a plateau that is reached after 8 hours. Fig. 3B shows corresponding values for region II. Here the amount of complex-bound acidic $\beta_2\text{m}$ exceeds that of the basic. In addition, no binding maximum is reached for any form. Note, that the 4-8 hours decrease of the acidic $\beta_2\text{m}$

complex (12 ng, Fig. 3A) almost equalizes the corresponding increase in Fig. 3B (14 ng). After the maximum binding in region I is reached, the total amount of bound β_{2m} is fairly constant for each form; 87-92 ng basic and 58-64 ng acidic β_{2m} was bound.

β_2M -containing complexes were obtained in both regions after re-chromatography of region I. This was also seen when pooled fractions corresponding to region I were concentrated and incubated separately with each β_{2m} form (not shown).

Different horse sera were analysed. The amount of bound β_{2m} varied somewhat between sera but the relative variations with the incubation time were same.

Determination of the affinity constant of the region I- β_{2m} complexes

After centrifugation (40 000 xg, 4 h) horse serum (30 ml) was chromatographed on Sephadex G-200 (5 x 135 cm) in 0.02 M Tris-HCl, pH 8.0 with 0.15 M NaCl and 0.02% NaN_3 . Fractions were combined in the way the arrows in Fig. 2 indicate and concentrated to about a third of the original serum volume. Centrifugation of serum did not affect the β_{2m} -interaction (not shown).

Experiments to analyse the affinity constant according to Scatchard (1949) of β_{2m} and region I (first part) were undertaken for both forms. A Scatchard plot for the basic β_{2m} is shown in Fig. 4. The accessible amount of radiolabelled β_{2m} was estimated to 10% by titration of a constant amount of β_{2m} with varying amounts of chromatographed material. In the serum, that also was used for the data presented in Fig. 3^A, at three different occasions, the affinity constant was determined to 1.9 , 3.0 and $2.1 \cdot 10^{10} \cdot M^{-1}$. The number of binding sites were calculated respectively to 3.6 , 3.0 and $3.0 \cdot 10^{16}$ /litre serum.

Corresponding experiments with the acidic β_{2m} were unsuccessful. Only 1-2% of the radiolabelled β_{2m} was accessible for binding and too large amounts of gel filtered horse serum was required.

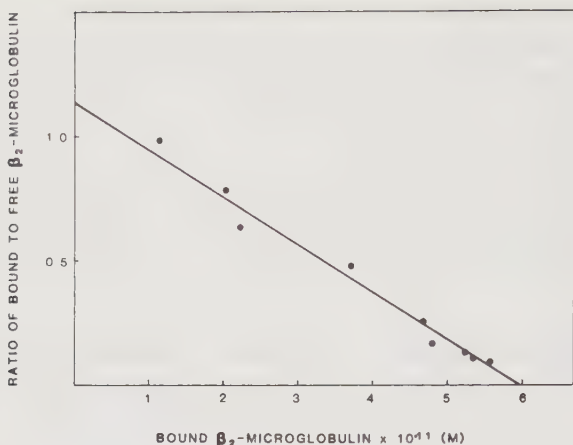


Fig. 4. Scatchard analysis of the binding of ^{125}I - $\beta_2\text{m}$ (basic) to the first part of region I in gel filtered horse serum. A volume of gel filtered material corresponding to $0.5\ \mu\text{l}$ horse serum was incubated 3 h, 37°C , with varying amounts of ^{125}I - $\beta_2\text{m}$ (basic form) in a final volume of 0.5 ml. After separation of bound and unbound $\beta_2\text{m}$ and correction for the inaccessible $\beta_2\text{m}$, the affinity constant and the number of binding sites were determined from the intercepts on the axis according to Scatchard (1949). In this experiment the accessible $\beta_2\text{m}$ was 10%, the affinity constant $1.9 \cdot 10^{10} \cdot \text{M}^{-1}$ and the number of binding sites $3.6 \cdot 10^{16}/\text{l}$ horse serum.

Binding of guinea-pig $\beta_2\text{m}$ to mouse serum

Mouse serum also contains $\beta_2\text{m}$ -containing complexes in region I (Natori *et al.*, 1976; Kvist and Peterson 1978) that bind exogenously added $\beta_2\text{m}$ (Lögberg *et al.*, 1981 b; Ramanathan *et al.*, 1982). Thus incubation for 4 h, 37°C , with each guinea-pig $\beta_2\text{m}$ form was performed to see whether the difference in binding was found only in horse serum.

The gel filtration $\beta_2\text{m}$ -binding profile in mouse serum differs somewhat from that of horse serum; region II contains, after 4 h incubation, the main part of the bound $\beta_2\text{m}$ - also the basic form - and coincides completely with the third protein peak.

The result of the binding experiment is presented in TABLE I and clearly shows a difference in binding capacity between the two forms in region I.

The difference is of the same magnitude as in horse serum. The initial incubations were made with previously lyophilized mouse serum. In this case hardly any β_2m was found in region I and no differences between the two forms were seen.

TABLE I. Binding of the two guinea-pig β_2m forms by mouse serum

β_2m added	β_2 -microglobulin (ng, (%) ^a)			
	I ^b	II	III	total
Basic	64 (29)	93 (42)	63 (29)	220 (100)
Acidic	39 (18)	98 (45)	82 (37)	219 (100)

β_2m of either form was incubated in mouse serum for 4 h, 37°C and then gel filtered on Sephadex G-200. The amount of β_2m in each region was calculated.

a) % of total β_2m in parenthesis

b) See Fig. 2 for definition of region I-III.

2

DISCUSSION

Much of later years research on β_2m has focused on the interaction between β_2m and MHC Class I antigens. In addition to the binding studies of isolated HLA-A,B,C antigens (or the equivalents in other species) and homologous or heterologous β_2m (Hyafil and Strominger 1979; Lögdberg *et al.*, 1980; Schmidt *et al.*, 1981; Church *et al.*, 1982; Parham 1983), the binding of β_2m to cell surface structures (Hester *et al.*, 1979; Sege *et al.*, 1979; Lögdberg *et al.*, 1981 a; Bernabeu *et al.*, 1984; Kefford *et al.*, 1984) or serum substances (Lögdberg *et al.*, 1981 b; Vincent *et al.*, 1981; Ramanathan *et al.*, 1982) have also been extensively studied. It is believed that on cell surfaces and in serum the β_2m -binding molecules are identical or related to MHC Class I antigens.

Although a substantial amount of data has accumulated around the circumstances for the interactions, not much is known about the structural parts of β_2m that are involved in the interaction.

In this study I have quantitatively compared the interaction between the two forms of guinea-pig β_2m and heterologous sera. The forms differ structurally in that one of the forms (acidic) lacks a C-terminal lysyl-residue (Cig  n, to be published).

A prerequisite, when to make a quantitative comparison of this kind is that the antibodies, used in the radioimmunoassay, react in a similar way with both β_2m components. This seemed to be the case as plots of serially diluted purified, unlabelled antigens were parallel (Fig. 1).

Serum from horse was chosen for the incubations as it is known from earlier studies that guinea-pig β_2m interacts with substances that on gel filtration elute in two regions in easily detected amounts (L  gdberg *et al.*, 1981 b). Also, large quantities of the same serum were available.

The total amounts of recovered β_2m were similar in all gel filtrations of β_2m /horse serum incubations, whereby it was possible to make a comparison between the two components.

Three major points were noted in the binding studies with horse serum: (1) less acidic than basic β_2m was found in region I, regardless of incubation time; (2) equal amounts or more acidic than basic β_2m were detected in region II; (3) the total amount of β_2m in complexes was, for each form, fairly constant, but in all cases less acidic β_2m was bound. Binding experiments with mouse serum also revealed that less acidic than basic β_2m was bound.

It is known that complexes in region I that bind β_2m , decompose to complexes detected in region II after re-chromatography (Kvist and Peterson, 1978; L  gdberg *et al.*, 1981 b; Ramanathan *et al.*, 1982; present paper). Thus dissociation of region I complexes explains the relatively slow binding of either form to region II, but it is unclear whether it accounts for all the β_2m recovered in this region. The results also indicate that region I complexes decompose more easily when acidic β_2m is bound. The possibility of a molecule in this region that rather binds acidic than basic β_2m may also be considered.

A lower affinity constant could explain the lesser amount bound acidic β_2m . Experiments to determine the affinity constant between region I (first part) and acidic β_2m were, however, unsuccessful. Hardly any

acidic β_2m beyond background levels was bound. In contrast, about 10 % of basic β_2m was accessible in these experiments. This is a rather small fraction but is in agreement with what has been reported (Lögdberg and Björck, 1983). It is believed that the iodination of certain tyrosine residues in human β_2m blocks its binding to HLA-B7 (Parker and Strominger, 1983). A mechanism of this kind could explain the small fraction ^{125}I -labelled guinea-pig β_2m available to horse serum. It can not be excluded that the acidic β_2m is more prone to get damaged during the radiolabelling process as indicated by the radioimmunoassay titration analysis (see above) or else get a slightly changed conformation that explains the low binding of ^{125}I -labelled acidic β_2m . Another possibility may be that a crucial tyrosine residue more readily become iodinated in acidic than in basic β_2m . Part of the ^{125}I -acidic β_2m may also escape detection. Region II complexes are not precipitated by 10 % polyethylene glycol used to separate bound and free β_2m (personal observation). The indication of a larger tendency for region I complexes to dissociate when acidic β_2m is bound may support this concept.

Although no comparable Scatchard data were obtainable there still exists a difference in binding capacity between the two guinea-pig β_2m forms. This is most probably a result of their structural difference (Cigén, to be published). Nothing is known about the orientation of the C-terminal amino acid in guinea-pig β_2m . As it was difficult to release the C-terminal amino acid unless the protein was reduced, alkylated and kept in detergent solution, it is conceivable that the amino acid, although hydrophilic, is hidden from the surface of the protein.

It is still too early to tell whether the lysine itself is a part of a binding site or not. Electrostatic forces have been reported to be responsible for some of the interaction forces between human β_2m and molecules in cod serum (Lögdberg and Björck, 1983). The lysyl-residue could be essential for keeping the tertiary conformation necessary for the interaction with other molecules. In any case more structural information of guinea-pig β_2m is needed to clear the picture of the interaction events.

It has been discussed that the amino acids in β_2m involved in the interaction with other molecules (i.e. MHC Class I antigens) should be the most evolutionary conserved amino acids in the protein. The interspecies exchange of β_2m in β_2m -containing complexes also favours this idea (cf Lögdberg *et. al.*, 1980). So far the C-terminal of β_2m in guinea-pigs is unique to this animal. Thus it does not constitute one of the well

conserved amino acids. However, the number of species in which the C-terminal amino acid is known is still limited (Cunningham *et. al.*, 1973; Gates *et. al.*, 1979; Gates *et. al.*, 1981; Lögberg, 1982; Groves and Greenberg, 1982).

The chemical nature of the β_2m -binding substances in horse serum is unknown. In mouse serum at least two different β_2m -binding molecules have been detected. Both molecules resemble Class I molecules but lack alloantigenic reactivity (Natori *et. al.*, 1976; Kvist and Peterson, 1978; Ramanathan *et. al.*, 1982; Maloy *et al.*, 1984). Thus, one could speculate in that the β_2m -binding molecules in horse serum are related to MHC Class I molecules.

This taken into account, together with the recent observation that no acidic guinea-pig β_2m was detected on membranes from liver tissue (Cigén, 1983), the investigation of the capacity of the two forms to interact with purified Class I molecules would be of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Faculty, University of Lund, Sweden and Konung Gustav V:s 80-årsfond, Sweden.

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